Androgens enhance adult hippocampal neurogenesis in males but not females in an age-dependent manner

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Abstract

Androgens (testosterone and dihydrotestosterone) increase adult hippocampal neurogenesis by increasing new neuron survival in male rats and mice via an androgen receptor pathway, but it is not known whether androgens regulate neurogenesis in females and whether the effect is age-dependent. We investigated the effects of dihydrotestosterone, a potent androgen, on neurogenesis in adult and middle-aged males and females. Rats were gonadectomized and injected with the DNA synthesis marker, bromodeoxyuridine (BrdU). The following day rats began receiving daily injections of oil or DHT for 30 days. We evaluated cell proliferation (Ki67) and new neuron survival (BrdU and BrdU/NeuN) in the hippocampus of male and female rats using immunohistochemistry. As expected, DHT increased new neuron survival in young males but surprisingly not in middle-aged male rats. In females, DHT did not significantly affect adult neurogenesis in young or middle age. Our results indicate that DHT regulates adult hippocampal neurogenesis in a sex- and age-dependent manner.

Main text

Neurogenesis, the production of new neurons, in the hippocampus continues through the life span of most mammals studied to date (Kempermann et al., 2018). Sex hormones (estrogens and androgens) regulate different aspects of hippocampal neurogenesis: e.g. proliferation and/or survival of these new neurons in rodents (reviewed in Duarte-Guterman et al., 2015b; Mahmoud et al., 2016). There is also evidence of sex differences in how hormones regulate neurogenesis. For example, estradiol regulates new neuron survival and cell proliferation in female but not male rodents (Barker and Galea, 2008). We have previously shown that androgens (testosterone and dihydrotestosterone) increase the survival of new neurons but not cell proliferation in the hippocampus of male rats via an androgen receptor (AR) pathway (Spritzer and Galea, 2007; Hamson et al., 2013) and mice (Swift-Gallant et al., 2018). However it is not known whether androgens regulate any aspects of adult neurogenesis in females. In addition to sex, age can also modulate the effects of hormones on hippocampal neurogenesis. In middle age, the hippocampus loses its ability to respond to estrogens in female rats. For instance, estradiol increases cell proliferation in the hippocampus in young but not middle-aged nulliparous female rats (Chiba et al., 2007; Barha and Galea, 2011). The objective of this study was to investigate the effects of dihydrotestosterone (DHT) on hippocampal neurogenesis (proliferation and new neuron survival) in young and middle-aged male and female rats.

At 2 months (~70 days old, young) and 11-12 months of age (middle-aged), gonadectomized male and female Sprague–Dawley rats received a single intraperitoneal injection of bromodeoxyuridine (BrdU; 200mg/kg) to label dividing cells and their progeny (Hamson et al., 2013). The following day, males and females were injected subcutaneously with either 0.25mg dihydrotestosterone (DHT in 0.1ml of sesame oil) or an equivalent volume of

sesame oil for 30 days. Twenty-four hours after the final injection, animals were overdosed with sodium pentobartitol and brains perfused and collected for BrdU (survival of 30 day old cells), Ki67 (cell proliferation marker), androgen receptor (AR), and BrdU/NeuN (new neurons using NeuN, marker for mature neurons) immunohistochemistry (Hamson et al., 2013; Swift-Gallant et al., 2018). Thus, in this experiment, BrdU+ cells were 30 day-old daughter cells from progenitor cells that had been synthesizing DNA for a 2-hour period 31 days before euthanasia. All protocols were approved by the Animal Care Committee at the University of British Columbia and conformed to the guidelines set out by the Canadian Council for Animal Care.

A researcher blind to experimental conditions counted BrdU+ and Ki67-immunoreactive (ir) cells in the entire rostrocaudal extent of the granule cell layer (GCL) including the sub granular zone, defined as the 50 µm band between the GCL and the hilus. An estimate of the total number of cells in the GCL and hilus was calculated by multiplying the number of cells counted by 10 to account for the fact that we used 1/10 series of sections (Tanapat et al., 2005; Spritzer and Galea, 2007; Swift-Gallant et al., 2018). GCL and hilus volumes were quantified from digitized images using Cavelier's principle, multiplying the sum of the area of each section by the section thickness (40 µm Gundersen et al., 1988). Androgen receptor immunoreactivity was characterized using a relative scale: robust (+++), intermediate (++), light (+), or absent (0) in the GCL, CA1, and CA3 regions in DHT and oil treated groups as we have done before (Hamson et al., 2013). To determine whether BrdU+ cells were of a neuronal phenotype, in a subset of brains, all BrdU+ cells within a section were examined for co-labeling with NeuN (neuronal marker) for 50 cells (young adult group) or all cells (middle-aged group) were phenotyped. All analyses were performed using Statistica v.8.0 (StatSoft Inc, Tulsa, OK). Volume of the dentate gyrus (GCL and hilus) and density of BrdU+ cells (total cells per unit

volume) were analyzed using repeated measures analysis of variance (ANOVA) with age (young, middle-aged), sex (male, female), and treatment (DHT, oil) as between subjects factors, and region (GCL, hilus) as a within subjects factor. Density of Ki67-ir cells and proportion of BrdU/NeuN co-labeled cells were analyzed using ANOVA with age (young, middle-aged), sex (male, female), and treatment (DHT, oil) as between subjects factors. When appropriate, post-hoc analysis used the Neuman-Keul's procedure. Test statistics were considered significant if $p \leq 0.05$.

Regardless of age and treatment, the GCL and hilus were larger in males compared to females (main effect of sex; $F(1,42)=4.42$; $P<0.05$; Table 1). The volume of the GCL and hilus increased with age irrespective of sex and treatment (main effect of age; $F(1,42)=47.37$; $P<0.0001$). In the case of the hilus, the volume increased with aging more so in males than in females (interaction between region, age, and sex; $F(1,42)=20.20$; $P<0.0001$). As expected, the volume of the hilus was larger than the volume of the GCL (main effect of region; $F(1,44)=1450.14$; $P<0.0001$). Treatment with DHT did not significantly affect GCL or hilus volumes (all P's>0.08). To account for the sex differences in GCL and hilus volumes, we present BrdU and Ki67 counts as densities (total cells per unit volume).

DHT treatment increased the density of BrdU+ cells in the GCL in young males ($P<0.001$) but not in young females or middle-aged animals of both sexes (all P's>0.9; interaction between age, sex, treatment and region; $F(1,42)=4.03$; $P=0.05$; Figure 1). The density of BrdU+ cells in the GCL was significantly higher in the young compared to the middle-aged rats irrespective of sex and treatment, as expected (main effect of age; $F(1,42)=647.85$; $P<0.0001$). In the hilus, the density of BrdU+ cells was not significantly affected by age, sex or treatment (all P's>0.9). To determine how many BrdU+ cells were neurons, we examined the

colabelling of BrdU and NeuN (a neuronal marker; BrdU/NeuN) in the GCL. Aging decreased the proportion of BrdU/NeuN colabeled cells in the dentate gyrus (main effects of age; $F(1,39)=27.98$; $P<0.0001$). In the young group, sex and treatment did not affect the percent of BrdU/NeuN colabeled cells (all P's>0.18; Table 2). In middle age, DHT treatment increases the proportion of BrdU/NeuN cells irrespective of sex (interaction between age and treatment; $F(1,39)=4.77$; $P=0.03$).

DHT treatment did not affect the density of Ki67-ir cells in young or middle age males and females (all P's>0.25; Figure 1). However, as expected, there were more Ki67-ir cells in the GCL of the young compared to middle-aged rats (main effect of age; $F(1,43)=97.18$; $P<0.0001$) irrespective of sex and treatment.

Finally, we performed a qualitative analysis on AR expression in the hippocampus. In gonadectomized oil treated animals, AR-ir cells were absent throughout the GCL in young and middle-aged male and female rats. In the CA1, we found low to intermediate levels of AR-ir and in the CA3 low to absent levels of AR-ir in young and middle-aged rats of both sexes. DHT treatment increased the expression of AR in the CA1 to intermediate or robust levels in both sexes (Table 3; Figure 1).

In the present study, we found that chronic (30 days) DHT increased new neuron survival but not cell proliferation in the hippocampus of gonadectomized young adult male rats, consistent with our previous research in male rats and mice (Spritzer and Galea, 2007; Hamson et al., 2013; Swift-Gallant et al., 2018). This is also in line with previous work showing that castration decreases new neuron survival 24-30 days after BrdU injection but has no effect on cell proliferation (Spritzer and Galea, 2007; Wainwright et al., 2011). Shorter testosterone treatment (3, 15 or 21 days) has no effect on hippocampal neurogenesis (Zhang et al., 2010;

Spritzer et al., 2011; Carrier and Kabbaj, 2012; Wainwright et al., 2016) indicating that a longer exposure (30 days) to androgens is required to increase neurogenesis in the hippocampus at least in physiological doses as higher doses can decrease neurogenesis (Brännvall et al., 2005; Zhang et al., 2014). Thus, collectively while longer term exposure to androgens increases new neuron survival in the dentate gyrus, androgens do not appear to influence cell proliferation in male rats (this study; Spritzer and Galea, 2007; Hamson et al., 2013), mice (Swift-Gallant et al., 2018), or voles (Fowler et al., 2003).

In contrast to young adult males, DHT did not affect new neuron survival or cell proliferation in gonadectomized young adult female rats. We previously found that estradiol modulates cell proliferation and new neuron survival in young adult female rats (Ormerod et al., 2003; Mazzucco et al., 2006; Barker and Galea, 2008; Barha et al., 2009; Duarte-Guterman et al., 2015a) but has no effect on neurogenesis in adult male rats (Spritzer and Galea, 2007; Barker and Galea, 2008). Together this suggests that sex steroids have sex-specific effects on hippocampal neurogenesis with androgens modulating neurogenesis in young adult males and estrogens modulating neurogenesis in young adult females. While these findings may not seem surprising, it is important to understand that both sexes have ARs and estrogen receptors (ERs) but these receptors are responding to respective hormones in a sex-specific way to modulate neurogenesis in the hippocampus.

Perhaps surprisingly, the effect of DHT to modulate survival of new neurons was absent in middle-aged males and females. This effect is consistent with the work of Moser et al. (2019) who found that testosterone did not affect the number of immature neurons (using doublecortin), in middle-aged (13 months) or aged (23 months) male rats. This would suggest that with aging, the dentate gyrus loses its ability to respond to sex steroids. Indeed, in females, estradiol

increases cell proliferation in young but not middle-aged rats (Barha et al., 2009; Barha and Galea, 2011). Intriguingly in females, previous reproductive experience (pregnancy and motherhood) can rescue the hippocampus' response to estrogens later in middle-age, as acute estrogens increased cell proliferation in multiparous rats (Barha and Galea, 2011). Thus, it is possible that experience, reproductive or otherwise, may restore the ability of androgens to upregulate hippocampal neurogenesis in males in middle age. However, Moser et al. (2019) did not see an influence of high fat diet on the androgen modulation of neurogenesis. Intriguingly in our study, the proportion of BrdU/NeuN colabelled cells was affected by age and treatment. DHT increased the proportion of BrdU/NeuN colabelled cells, an effect mostly driven by the middle-aged females, and overall as expected, the proportion of new cells that express mature neuronal protein decreased with age. In middle-aged female mice letrozole, an aromatase inhibitor blocking the conversion of androgens to estrogens, increases hippocampal neurogenesis (using the immature marker doublecortin; Chaiton et al., 2018). Together with our findings, this suggests that the hippocampus may still be able to respond to sex steroid hormones in middle age, an effect that varies by sex as in both studies middle-aged females showed increased neurogenesis levels with androgens.

We found that ARs were expressed in the CA1 and CA3 regions of the hippocampus. We did not find expression of ARs in the granule cell layer in male rats and this is consistent with previous research (Kerr et al., 1995; Xiao and Jordan, 2002; Hamson et al., 2013); although there is conflicting evidence which may be due to strain and species differences. In Wistar male rats, Moghadami et al. (2016) and Brännvall et al. (2005) found AR expression in the granule cell layer in young male rats. In gonadectomized male mice, ARs are not expressed in the granule cell layer but in males receiving DHT treatment, granule cells express AR (Swift-Gallant et al.,

218). In other rat strains (Bruce-Spruce Long-Evans, Fischer 344 and Sprague Dawley), ARs are not expressed in the granule cell layer (Xiao and Jordan 2002; Kerr et al., 1995; Hamson et al., 2013). We also found that DHT increases the expression of ARs in the CA1 in males in line with previous research in young male rats (Hamson et al., 2013) and male mice (Swift-Gallant et al., 2018). In females and males, 2 days of testosterone treatment also showed increased AR expression in the CA1 region (Xiao and Jordan, 2002) but to our knowledge no other studies have investigated AR expression after chronic DHT treatment in females. Previous work has found that androgens increase neurogenesis via the androgen receptor (AR). Blocking the AR with flutamide abolishes the increase in new neuron survival in male rats (Hamson et al., 2013). However, in male rats and mice, ARs are not found in immature neurons (doublecortin expressing cells) in the granule cell layer of the hippocampus (Hamson et al., 2013; Swift-Gallant et al., 2018). We have previously proposed that one mechanism of action is that androgens bind to ARs in the CA3 region and this initiates a retrograde response of a survival factor that targets newborn neurons in the granule cell layer (Galea et al., 2013). In the current study, we found ARs were expressed in the CA1 and CA3 regions in young and middle-aged male and female rats and their expression increases after DHT treatment in both sexes at both ages. However, we only observed an effect of DHT on new neuron survival in young males. In young females and middle-aged males and females, ARs are expressed in the CA1 and CA3 but binding of DHT to these ARs does not result in the modulation of neurogenesis, possibly indicating different downstream mechanisms of bound AR to modulate neurogenesis in young adult females, and middle-aged males. Interestingly, we found higher expression of AR in the CA1 of middle-aged compared to young rats of both sexes. AR mRNA expression increases in the hippocampus with aging in males (Kerr et al., 1995). DHT did not increase new neuron

survival in mice overexpressing AR in the CA1, indicating that high doses of AR result in an abolished response (Swift-Gallant et al., 2018). It is possible that the increase in AR expression with aging is responsible for the lack of DHT mediated increase in new neuron survival.

To summarize, DHT increased survival of new neurons in young adult male rats but not in female or middle-aged male and female rats. However, DHT treatment increased the proportion of surviving cells expressing the mature neuronal protein in middle-aged females and AR expression in both sexes, regardless of age, suggesting that ARs have the potential to respond to DHT. Our current work together with our previous research (reviewed in Mahmoud et al., 2016) indicate that androgens and estrogens have sex-specific and age-specific effects on hippocampal neurogenesis.

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FIGURES

Figure 1. Chronic dihydrotestosterone (DHT) treatment increases the density of BrdU+ cells in the dentate gyrus of young male adult rats but has no effects in middle-aged males and young and middle-aged female rats. (**A**) Photomicrographs of a representative section of dentate gyrus with the granule cell layer (GCL), hilus, CA1 and CA3 regions and representative BrdU+ and Ki67-ir cells in the subgranular zone. (**B**) Representative images of androgen receptor (AR) expression in the CA1 region of the hippocampus in male and female rats treated with oil or DHT. AR is lowly expressed in the CA1 of gonadectomized oil treated animals. DHT increases the expression of AR in both male and female rats. (**C**) Mean ± SEM total number of BrdU+ cells in the granule cell layer of the dentate gyrus in young and middle-aged male and female rats. In young males, DHT increased the density of BrdU+ cells relative to the oil treatment group ($P < 0.001$). (**D**) Mean ± SEM total number of Ki67-ir cells in the granule cell layer of the dentate gyrus in young and middle-aged male and female rats. Chronic DHT treatment did not affect cell proliferation in the hippocampus of young or middle-aged male and female rats. Circles represent individual data points.

TABLES

Table 1. Mean (SEM) volume of the granule cell layer (GCL) and the hilus (mm^3) and BrdU density in the hilus in gonadectomized young and middle-aged male and female rats treated with oil or dihydrotestosterone (DHT). Males had a larger volume of GCL and hilus and the volume of the hilus increased with aging more so in males than females. DHT treatment did not have an effect on the volume of the GCL and hilus.

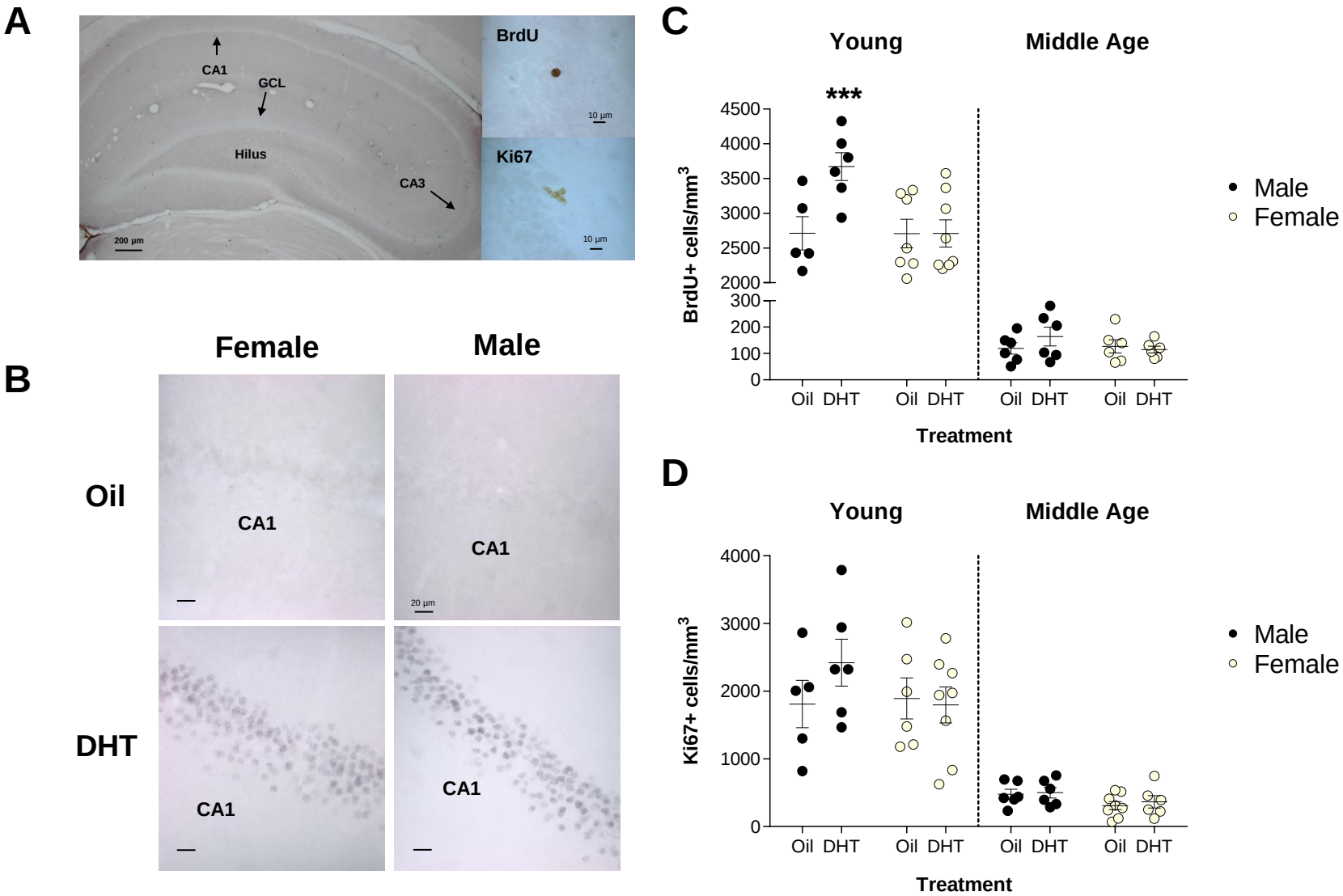
Age	**Sex and Treatment**	**Sample size**	**GCL volume (mm^3)**	**Hilus volume (mm^3)**	**Hilus BrdU density (per mm^3)**
Young	*Females*				
	Oil	7	2.51 (0.12)	6.09 (0.19)	85.14 (15.34)
	DHT	8	2.46 (0.08)	6.00 (0.28)	76.06 (7.06)
	Males				
	Oil	5	2.31 (0.19)	5.32 (0.49)	96.18 (23.29)
	DHT	6	2.62 (0.21)	5.37 (0.51)	134.70 (17.38)
Middle Age	*Females*				
	Oil	6	2.33 (0.09)	7.67 (0.42)	20.69 (7.89)
	DHT	6	2.45 (0.15)	7.33 (0.28)	6.40 (2.49)
	Males				
	Oil	6	2.94 (0.25)	9.05 (0.79)	6.06 (1.41)
	DHT	6	2.87 (0.22)	10.05 (0.60)	4.65 (1.63)

Table 2. Mean (SEM) percentage of cells co-expressing BrdU and NeuN in the GCL in gonadectomized young and middle-aged male and female rats treated with oil or dihydrotestosterone (DHT). The proportion of BrdU+ cells colabelled with NeuN was significantly higher in young compared to middle-aged animals. In middle age, DHT increases the proportion of BrdU/NeuN colabelled cells relative to the oil treatment group.

Age	Sex and Treatment	Sample size	BrdU/NeuN %
Young	*Females*		
	Oil	5	87.89 (2.27)
	DHT	8	87.16 (1.10)
	Males		
	Oil	4	88.68 (1.94)
	DHT	5	84.24 (3.12)
Middle Age	*Females*		
	Oil	6	63.80 (3.43)
	DHT	6	81.32 (5.82)
	Males		
	Oil	6	67.03 (5.39)
	DHT	6	70.88 (7.00)

Table 3. Androgen receptor (AR) expression in the hippocampus using a relative rating scaling: absent (0), light (+), intermediate (++), and robust (+++) in the granule cell layer (GCL), CA1, and CA3 regions in young and middle-aged male and female rats treated with oil or dihydrotestosterone (DHT).

Age	**Sex and Treatment**	**AR expression**		
		GCL	**CA1**	**CA3**
Young	*Females*			
	Oil	0	0/+	0
	DHT	0	+/++	0/+
	Males			
	Oil	0	0	0
	DHT	0	+/++	0/+
Middle Age	*Females*			
	Oil	0	+/++	+
	DHT	0	+++	+
	Males			
	Oil	0	+/++	0/+
	DHT	0	+++	0/+


A
CA1
GCL
Hilus
CA3
200 µm
BrdU
10 µm
Ki67
10 µm
B
Female
Male
Oil
DHT
CA1
CA1
20 µm
CA1
CA1
C
Young
Middle Age
***
BrdU+ cells/mm3
4500
4000
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200
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Male
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Ki67+ cells/mm3
4000
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Oil DHT Oil DHT Oil DHT Oil DHT
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