

Peripubertal aromatase inhibition in male rats has adverse long-term effects on bone strength and growth and induces prostatic hyperplasia

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Abstract

Aromatase inhibitors have been increasingly used in boys with growth retardation to prolong the duration of growth and increase final height. Multiple important roles of oestrogen in males point to potential adverse effects of this strategy. Although the deleterious effects of aromatase deficiency in early childhood and adulthood are well documented, there is limited information about the potential long-term adverse effects of peripubertal aromatase inhibition. To address this issue, we evaluated short-term and long-term effects of peripubertal aromatase inhibition in an animal model. Peripubertal male Wistar rats were treated with aromatase inhibitor letrozole or placebo and followed until adulthood. Letrozole treatment caused sustained reduction in bone

strength and alteration in skeletal geometry, lowering of IGF1 levels, inhibition of growth resulting in significantly lower weight and length of treated animals and development of focal prostatic hyperplasia. Our observation of adverse long-term effects after peripubertal male rats were exposed to aromatase inhibitors highlights the need for further characterisation of long-term adverse effects of aromatase inhibitors in peripubertal boys before further widespread use is accepted. Furthermore, this suggests the need to develop more selective oestrogen inhibition strategies in order to inhibit oestrogen action on the growth plate, while beneficial effects in other tissues are preserved.

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Introduction

The crucial role of oestrogen in mediating epiphyseal fusion in males was established by classical reports of men with defective oestrogen synthesis (aromatase deficiency; Morishima *et al.* 1995) and action (oestrogen receptor α deletion; Smith *et al.* 1994). These men in their twenties had tall stature and open epiphyses despite normal pubertal development and testosterone levels, indicating that oestrogen, and not testosterone, was responsible for epiphyseal fusion in men. This realisation prompted the development of a novel treatment for boys with short stature using aromatase inhibitors, which block oestrogen production in order to prolong the duration of growth and to increase final height. Beneficial effects of aromatase inhibitors on growth have been reported in boys with constitutional delay of puberty and growth, idiopathic short stature and GH deficiency (Wickman *et al.* 2001, Mauras *et al.* 2004, 2008, Hero *et al.* 2005, 2006b). The ease of administration, low cost and lack of overt toxicity of aromatase inhibitors make them a potential

attractive strategy for treatment of boys with idiopathic short stature. This has led to a significant increase in the use of aromatase inhibitors in short boys, especially in North America and Europe (Shulman *et al.* 2008).

The use of aromatase inhibitors in young boys, however, remains 'off label', as these agents were primarily developed for the treatment of hormone-responsive breast cancer and preclinical studies were not performed in peripubertal males. Short-term follow-up (2–3 years) of boys treated with aromatase inhibitors has not shown significant adverse effects on areal bone density (Wickman *et al.* 2003), body composition (Hero *et al.* 2006a) or spermatogenesis (Mauras *et al.* 2005). However, these studies are limited by small sample size and short duration of follow-up. Importantly, many adverse effects of aromatase inhibition may not be manifested until adulthood. A fundamental assumption for the use of aromatase inhibitors in boys is that oestrogen deficiency has no deleterious consequences. However, this proposition is questioned by the important physiological role of oestrogen in males as illustrated by the development of osteoporosis

(Bilezikian *et al.* 1998, Oz *et al.* 2000), adiposity phenotype (Morishima *et al.* 1995, Jones *et al.* 2000, Takeda *et al.* 2003) and impaired spermatogenesis (Carani *et al.* 1997, Robertson *et al.* 1999, Murata *et al.* 2002) in aromatase-deficient men and mice. These models, however, indicate the effects of long-standing oestrogen deficiency and are therefore not representative of selective peripubertal aromatase inhibition. Deleterious skeletal (Vanderschueren *et al.* 1997) and testicular (Turner *et al.* 2000) effects in adult Wistar rats treated with aromatase inhibitors suggest the potential of adverse effects of transient aromatase deficiency. However, the effects of transient aromatase inhibition in peripubertal males remain largely unexplored. We therefore developed an animal model to study these effects. In our study, peripubertal male Wistar rats were treated with letrozole, a highly selective, non-steroidal aromatase inhibitor. They were then followed up until early adulthood. The use of this model allowed us to study the long-term consequences of aromatase inhibition in peripubertal males.

Materials and Methods

The study was conducted at the large animal facility of the Royal Children's Hospital, Melbourne, Australia, in accordance with the Australian Code of Practice for the care and use of animals for scientific purposes, 2004. Ethical approval was obtained from the Animal Ethics Committee of the Royal Children's Hospital (AEC number 554).

Animals

Fifty 27-day-old male Wistar rats were obtained from Central Animal Services, Monash University, Clayton, Australia. After 3 days of acclimatisation, they were randomised to treatment ($n=25$) and control ($n=25$) groups. The animals were housed individually in similar environmental conditions (reverse day–night cycle with 12 h day and night, temperature 24 °C). They were fed oestrogen-free diet (AIN93M, Specialty Feeds, Perth, Western Australia, Australia) to avoid the confounding effect of environmental oestrogen exposure. No dietary- or water restrictions were imposed. The animals were inspected daily for well-being and weighed bi-weekly. Procedures were performed under anaesthesia induced by a combination of i.p. xylazine (10 mg/kg) and ketamine (100 mg/kg).

Intervention

The treatment group received letrozole orally (Femara, Novartis group, 1 mg/kg per day dissolved in 10% gelatin; Nolan & Levy 2006). The gelatin preparation was consumed avidly by the rats. They were treated from the peripubertal age of 30 days for 60 days until the age of 90 days. This is roughly equivalent of ages 10–18 years in human terms. The control animals received gelatin vehicle alone.

The animals were followed for an additional 90 days after completion of treatment until adulthood at the age of 180 days, which equates to around 30 human years of age (Fig. 1). Blood was obtained from the tail vein at the start of study (day 0), at the end of treatment (day 60) and at the end of study (day 150). Serum was separated and stored at -70 °C until analysis. The animals were culled by injecting pentobarbital (0.5 ml of 340 mg/ml; Virbac Animal Health, NSW, Australia).

Blood assays

Blood glucose was measured using a Medisense Optimum glucometer (Abbott Laboratories); insulin-like growth factor 1 (IGF1) was measured using a rat/mouse IGF1 ELISA (Immunodiagnostic Systems Ltd (IDS), Fountain Hill, AZ, USA) with intra- and inter-assay coefficients of variation (CV) of 4.3–8.8 and 6.3–8.8% respectively. A rat/mouse GH kit (Linco Diagnostics, St Charles, MO, USA) was used for measurement of GH (intra-assay CV 1.7–4.3%; inter-assay CV 3.2–4.9%) on a single sample. Bone turnover was assessed with the bone formation marker serum osteocalcin (rat osteocalcin EIA kit, Biomedical Technology Inc., Stoughton, MA, USA; intra-assay variation 4%; inter-assay variation 7%) and the bone resorption marker C-terminal telopeptide α 1 chain of type I collagen (RATLAPS from IDS; intra-assay variation <10%, inter-assay variation <15%). LH and FSH were measured by RIA with the following iodinated preparations (iodinated using Iodogen reagent (Sigma) and anti-sera: recombinant (r) FSH 1–8 and anti-rFSH-S-11, and rLH-1–9 and anti-rLH-S-10 (NIDDK, Bethesda, MD, USA)). The secondary antibody used in both RIAs was goat anti-rabbit IgG (GAR no. 12; Monash Institute of Reproduction and Development, Monash University, Melbourne, Australia), and the assay buffer was 0.01 M PBS containing 0.5% BSA (Sigma). All samples were measured in a single assay as described previously (O'Donnell *et al.* 1994). Serum levels of testosterone were determined using the DSL-4000-coated tube RIA as per the manufacturer's instructions (Diagnostic System Laboratories Inc., Webster, TX, USA). Insulin (intra-assay CV 1.15–3.65%; inter-assay CV 6.71–9.23%) levels were measured using kits from Linco Diagnostics.

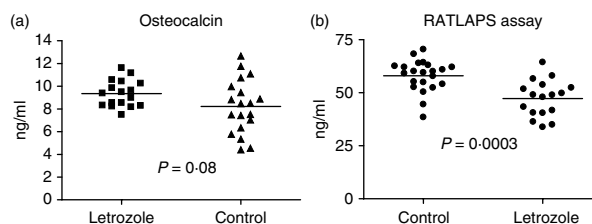


Figure 1 Results from bone turnover markers. (a) No significant difference in bone formation, as assessed by measuring serum osteocalcin levels, was observed between treated and non-treated animals. (b) Bone resorption, as assessed by RATLAPS assay, was significantly reduced in letrozole-treated animals as compared to controls.

Dual energy X ray absorptiometry

Dual energy X ray absorptiometry (DXA) was performed at the end of treatment (day 60) and at the end of study (day 150) using a QDR 4500A densitometer (Hologic Inc., Bedford, MA, USA) with dedicated small animal software (V9 L1 Rev A for acquisition and rat whole body V8.26a for analysis). Total mass, lean mass, fat mass and percentage of body fat were determined by whole body DXA. Tibial and crown–rump lengths were measured using the DXA image at both time points.

Peripheral quantitative computed tomography

Peripheral quantitative computed tomography (PQCT) of the tibia was performed at the end of treatment (day 60) and at the end of study (day 150) using an XCT Research SA+ densitometer (Stratec Medizintechnik GmbH, Pforzheim, Germany) by a single-blinded observer. Two CT slices were obtained at distances of 4 and 30% tibial length from the end of the left tibia to assess trabecular and cortical bone respectively. Contour mode 1 and peel mode 20 were used for analysis. A voxel size of 0.1 mm was selected. Cross-sectional bone area and volumetric bone density were calculated at both sites. Cortical thickness, periosteal circumference, endosteal circumference and stress–strain index were measured at the 30% site.

Prostate

The weight of individual lobes of the prostate was measured at the time of culling. Prostate was fixed in modified Bouin's solution, embedded in paraffin and cut into 5 µm sections as described earlier. Tissue sections were stained with haematoxylin and eosin to study morphological changes or subjected to immunohistological examination using antibodies to proliferating cell nuclear antigen (PCNA; PC10; Dako Corp., Carpinteria, CA, USA) or Apoptag.

Analyses of PCNA and apoptotic marker immunolocalisation were conducted using CAST software (version 2.1.4; Olympus Corp., Albertslund, Denmark) as described earlier (McPherson *et al.* 2001).

Testes

The weight of both testes was measured at the end of study. The testis was fixed in Bouin's solution and transferred into 70% ethanol. Testicular histology evaluation included assessment for disruption of spermatogenesis, and immunohistochemical analysis of cellular proliferation and apoptosis.

Statistical analysis

The study was powered to detect a difference of 1 SDS in stress–strain index with a power of 80% and a level of significance of 0.05. Seventeen animals were required in each group. We enrolled 25 animals in each group to allow for attrition. Data were expressed as mean ± s.d. SPSS version 10 (IL, USA) was used for statistical analysis. Quantitative parameters in the two groups were analysed using a two-tailed unpaired Student's *t*-test. A *P* value < 0.05 was considered significant.

Results

Of the 25 animals in each group, 5 (3 in the treatment group and 2 controls) died due to anaesthetic complications. Data for 22 animals in the treatment group and 23 animals in the control group are presented.

Skeleton

PQCT showed lower cross-sectional trabecular and cortical bone area in the treated animals (Table 1). This was associated with reduced periosteal and endosteal circumference and cortical thickness at the 30% tibial (cortical) site.

Table 1 Peripheral quantitative computed tomography skeletal parameters observed in the study

Parameter	End of treatment (day 60)		<i>P</i>	End of study (day 150)		<i>P</i>
	Treatment	Control		Treatment	Control	
Trabecular cross sectional area (mm ²)	15.5 ± 1.2	18.7 ± 0.9	<0.0001	18.0 ± 1.1	20.5 ± 1.6	<0.0001
Trabecular density (mg/mm ³)	403.3 ± 28.1	401.7 ± 37.6	0.87	448.2 ± 39.1	435.7 ± 51.9	0.37
Cortical cross sectional area (mm ²)	4.6 ± 0.3	5.8 ± 0.4	<0.0001	6.1 ± 0.4	7.4 ± 0.6	<0.0001
Cortical density (mg/mm ³)	1207.0 ± 16.7	1210.7 ± 28.4	0.62	1275.9 ± 13.1	1279.3 ± 12.7	0.38
Cortical thickness (mm)	0.50 ± 0.03	0.60 ± 0.03	<0.0001	0.70 ± 0.02	0.73 ± 0.03	0.002
Cortical thickness/TL (mm/cm)	0.138 ± 0.011	0.148 ± 0.011	0.005	0.166 ± 0.012	0.157 ± 0.009	0.01
Periosteal circumference (mm)	10.6 ± 0.4	11.8 ± 0.7	<0.0001	11.0 ± 0.5	12.4 ± 0.7	<0.0001
Periosteal circumference/TL (mm/cm)	2.84 ± 0.28	2.99 ± 0.21	0.05	2.62 ± 0.13	2.68 ± 0.18	0.23
Endosteal circumference (mm)	7.4 ± 0.5	8.1 ± 0.8	0.001	6.7 ± 0.6	7.8 ± 0.6	<0.0001
Endosteal circumference/TL (mm/cm)	1.97 ± 0.26	2.06 ± 0.20	0.23	1.58 ± 0.09	1.69 ± 0.15	0.006
Stress–strain index (mm ⁴)	4.0 ± 0.5	5.6 ± 0.5	<0.0001	5.1 ± 0.5	7.0 ± 1.1	<0.0001

TL, tibial length.

No difference was seen between the cortical and trabecular volumetric bone mineral density in the two groups. Treated animals had reduced linear skeletal growth as reflected by lower tibial length at the end of treatment (37.2 ± 2.6 vs 40.8 ± 1.7 mm, $P < 0.0001$) and at the end of study (40.8 ± 1.9 vs 46.4 ± 2.5 mm, $P < 0.0001$). When the skeletal geometry results were corrected for this reduced tibial length (Table 1), there was a significant reduction in cortical thickness/tibial length ratio and periosteal circumference/tibial length ratio at day 60 in the treated animals, whereas endosteal circumference to tibial length ratio showed a trend towards reduction. These skeletal changes resulted in a reduction in bone strength as reflected by lower stress-strain index in the treated animals.

At day 150, previously treated animals had a significantly decreased endosteal circumference/tibial length ratio, leading to an increased cortical thickness/tibial length ratio in the treatment group. The smaller bones, however, still showed a reduced stress-strain index at day 150.

Osteocalcin levels in treated animals were not significantly different from those measured in the control animals (letrozole ($n=17$) 9.352 ± 0.2841 ng/ml versus control ($n=19$) 8.220 ± 0.5438 ng/ml, $P=0.0835$; Fig. 1a), but letrozole-treated animals had lower RATLAPS levels (47.3 ± 8.6 vs 58.1 ± 7.4 ng/ml, $P=0.0003$; Fig. 1b) than controls at the end of treatment.

Growth

The two groups had similar weight at the start of study. Treated animals had lower relative weight gain during treatment compared to controls (179.1 ± 32.3 vs $354.3 \pm 57.8\%$, $P < 0.0001$) resulting in significantly lower weight than controls from day 10 onwards (Table 2 and Fig. 2a). This resulted in 37.5% lower weight in treated animals compared to controls at the end of treatment ($P < 0.0001$; Table 2). Both

Table 2 Growth parameters observed in the study

	Treatment ($n=22$)	Control ($n=23$)	<i>P</i>
Category			
Weight (g)			
Day 0	101.4 ± 13.5	100.2 ± 9.9	0.72
Day 60	284.2 ± 27.4	455.2 ± 43.8	< 0.0001
Day 150	391.3 ± 40.0	613.3 ± 80.0	< 0.0001
Weight gain % of baseline			
Day 1–60	179.1 ± 32.3	354.3 ± 57.8	< 0.0001
Day 61–150	37.7 ± 7.2	34.7 ± 7.9	0.31
Crown–rump length (cm)			
Day 60	17.5 ± 0.8	19.4 ± 0.7	< 0.0001
Day 150	18.7 ± 0.5	21.1 ± 0.6	< 0.0001
Tibial length			
Day 60	37.2 ± 2.6 (mm)	42.0 ± 1.7 (mm)	< 0.0001
Day 150	40.8 ± 1.9 (mm)	46.4 ± 2.5 (mm)	< 0.0001

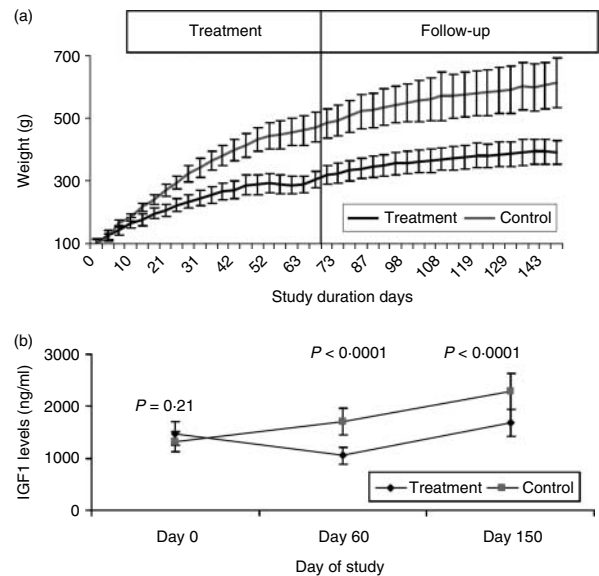


Figure 2 Effects of aromatase inhibitors on growth. (a) Treated animals had lower relative weight gain during treatment compared to controls (179.1 ± 32.3 vs $354.3 \pm 57.8\%$, $P < 0.0001$) resulting in significantly lower weight than controls from day 10 onwards. The weight of treated animals was 37.5% lower than controls. Both groups had similar relative weight gain (37.7 ± 7.2 vs $34.7 \pm 7.9\%$, $P=0.31$) in the follow-up period; the weight of the treated animals, however, remained 36.2% lower than controls at the end of study. (b) IGF1 levels were similar in the two groups at the start of study (1475 ± 95.87 vs 1316 ± 63.71 ng/ml, $P=0.21$); levels were lower in the treated animals at the end of treatment (1051.6 ± 268.8 vs 1706.2 ± 244.2 ng/ml, $P < 0.0001$) and at the end of study (1682 ± 40.53 vs 2282 ± 53.51 ng/ml, $P < 0.0001$).

groups had similar relative weight gain (37.7 ± 7.2 vs $34.7 \pm 7.9\%$, $P=0.31$) in the follow-up period; the weight of the treated animals, however, remained 36.2% lower than controls at the end of study ($P < 0.0001$). Linear growth was also compromised in the treatment group with lower crown–rump length and tibial length at the end of treatment and at the end of study ($P < 0.0001$ for both). IGF1 levels were similar in the two groups at the start of study (1475 ± 95.87 vs 1316 ± 63.71 ng/ml; $P=0.21$); levels were lower in the treated animals during treatment (1051.6 ± 268.8 vs 1706.2 ± 244.2 ng/ml, $P < 0.0001$) and at the end of study (1682 ± 40.53 vs 2282 ± 53.51 ng/ml, $P < 0.0001$; Fig. 2b). GH levels were unaffected by letrozole treatment (21.7 ± 22.9 vs 31.8 ± 22.4 ng/ml, $P=$ not significant).

Prostate

Letrozole-treated animals had larger anterior and dorsal lobes of prostate at the end of study when corrected for body weight (Table 3). No difference was noted in the weight of ventral and lateral prostate lobes corrected for body weight. Focal prostatic hyperplasia was observed in 15/19 treated animals (78.9%) with 4.8 ± 2.6 foci per tissue (Fig. 3a). None of the controls showed focal prostatic hyperplasia. A trend of

Table 3 Organ weights of prostate and testis at the end of study (expressed as g/100 g body weight)

Parameter	Treatment (n=22)	Control (n=23)	P
Ventral prostate (g/100 g)	0.099 ± 0.020	0.105 ± 0.021	0.28
Anterior prostate (g/100 g)	0.043 ± 0.009	0.035 ± 0.007	0.003
Lateral prostate (g/100 g)	0.036 ± 0.012	0.034 ± 0.009	0.57
Dorsal prostate (g/100 g)	0.060 ± 0.016	0.051 ± 0.012	0.08
Testis (g/100 g)	0.877 ± 0.117	0.634 ± 0.113	<0.0001

increased percentage of PCNA-positive epithelial cells was observed in the treated group compared to controls (4.27 ± 1.28 vs 2.17 ± 0.43 , $P=0.054$; Fig. 3b).

Reproductive measures

Testicular size was increased in the treated animals at the end of study, as reflected by higher testicular weight corrected for body weight (Table 3). Letrozole treatment was associated with a reduction in LH levels. The two groups had similar levels at the start of treatment (0.28 ± 0.24 vs 0.36 ± 0.26 ng/ml, $P=0.318$). LH levels were lower in the treatment group at day 60 (0.39 ± 0.33 vs 0.69 ± 0.27 ng/ml, $P<0.0001$) and were similar by the end of study (0.98 ± 0.32 vs 1.1 ± 0.57 ng/ml,

$P=0.409$). No differences were observed in the FSH (7.16 ± 1.99 vs 8.13 ± 1.98 ng/ml, $P=0.117$) and testosterone levels (2.06 ± 1.13 vs 1.82 ± 1.13 ng/ml, $P=0.482$) at the end of treatment in the two groups.

Body composition and metabolic parameters

Letrozole treatment did not affect the proportion of fat mass at the end of treatment (20.2 ± 4.8 vs $19.4 \pm 4.7\%$, $P=0.58$). Treated animals, however, had a lower proportion of body fat at the end of study (23.5 ± 5.8 vs $30.1 \pm 7.0\%$, $P<0.0001$). The insulin to glucose ratio was similar in the two groups during treatment (0.37 ± 0.15 vs 0.43 ± 0.26 , $P=0.38$).

Discussion

Our study has demonstrated significant long-term adverse effects of aromatase inhibition in peripubertal male rats. Peripubertal aromatase inhibition led to reduction in bone strength, impaired growth and prostatic hyperplasia. Importantly, these effects were long standing and persisted in adulthood.

The demonstration of persistent compromise in bone strength in the letrozole-treated animals was a critical finding in our study. This reiterates the observations of reduced bone strength in aromatase-deficient men (Bilezikian *et al.* 1998), aromatase knockout mice (Oz *et al.* 2000) and older male Wistar rats treated with aromatase inhibitor vorozole (Vanderschueren *et al.* 1997). In the first two models, however, aromatase inhibition was lifelong, whereas in the last model, aromatase inhibition only occurred in adulthood. In contrast, in our model, aromatase inhibition was limited to the peripubertal period, similar to its current usage in short boys. Abnormalities in skeletal geometry (reduced periosteal and endosteal circumference, bone length and cortical thickness), with resultant smaller, thinner bones, even when corrected for tibial length, were seen in letrozole-treated animals. However, bone mineral density was preserved. This suggests a direct inhibitory effect of the drug on periosteal apposition over and above the changes observed in linear growth. Treated animals showed catchup in bone size and periosteal apposition after discontinuation of treatment resulting in similar bone size corrected for tibial length in the two groups at last follow-up. The reduced stress-strain index observed at the end of study thus pertains to the small overall size of the bone. However, this smaller bone is still at increased risk of fracture compared to the larger bones observed in the control animals.

The change in bone geometry, but not density, indicates a differential role of oestrogen in the male skeleton with predominant effect on skeletal growth and cortical modelling and relatively minor effect on bone mass accrual. This is in concordance with the observations on growing wild-type and androgen receptor knockout male mice (Venken *et al.* 2006). The lack of variation in osteocalcin results and reduced bone

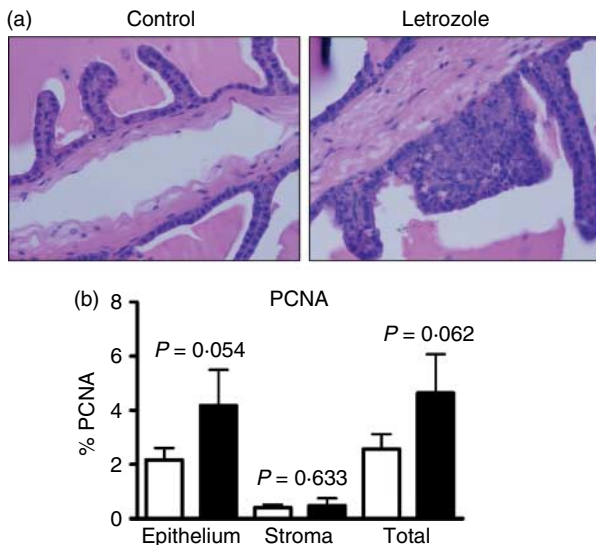


Figure 3 Effects of letrozole treatment on prostate. (a) Focal prostatic hyperplasia was observed in 15/19 treated animals (78.9%) with 4.8 ± 2.6 foci per tissue. None of the controls showed focal prostatic hyperplasia. (b) Letrozole-treated animals showed a trend of increased percentage of PCNA-positive epithelial cells compared to controls (4.27 ± 1.28 vs 2.17 ± 0.43 , $P=0.054$). Solid bars represent letrozole-treated animals and open bars represent controls (mean \pm s.d.).

resorption reflected by the RATLAPS assay between treated and untreated animals at this dose are consistent with the only published data in rats (Kumru *et al.* 2007). Given that osteocalcin reflects only one aspect of osteoblast function, it is possible that effects are being mediated on bone formation, which are not seen in the osteocalcin results.

Our findings highlight the limitations of the currently available evidence regarding bone safety in aromatase inhibitor-treated boys. These studies have assumed a lack of adverse skeletal effects by the demonstration of similar DXA-measured areal bone mineral density (BMD) and bone markers compared to controls, and have not used PQCT scans. Importantly, DXA does not provide robust information about skeletal geometry, the main site of oestrogen action. In three out of four of these studies, testosterone or GH, agents with direct anabolic effects on bone, was used in addition to aromatase inhibitors. Although aromatase inhibitors may be combined with GH and testosterone in boys with GH deficiency and constitutional delay of puberty and growth respectively, they are frequently used as a stand-alone treatment in idiopathic short stature, the most common indication in clinical practice. The observations of studies with combination treatment cannot be extrapolated to boys with idiopathic short stature. Another important factor in these studies is the relatively short follow-up duration (1–3 years). With fragility fracture the most important long-term indicator of bone health, the finding of vertebral compression in aromatase inhibitor-treated boys within 10 years of the initial treatment is particularly important (Dunkel 2009, Hero *et al.* 2009) and that this occurred despite normal DXA-measured areal BMD suggests that areal BMD is not an adequate predictor of bone strength in this setting.

A second significant observation of our study was the growth suppression induced by letrozole, with treated animals weighing 37.5% less than controls. This is similar to the observation of Vandescheuren *et al.* (1997) showing 16% lower weight in vorozole-treated growing Wistar rats, and Turner *et al.* (2000) showing 24% lower weight in anastrozole-treated adult male rats. Intriguingly, Eshet *et al.* (2004) observed increased weight gain in male mice treated with letrozole for 10 days. This is consistent with reports in female letrozole-treated rats, which showed weight gain and changes consistent with polycystic ovarian syndrome (Manneras *et al.* 2007). Our study examining male rats only is not able to shed further light on the discrepancy between reports in different genders. Importantly, impairment in weight gain in males was evident only after 2 weeks of treatment in our and other studies, as distinct from females. Longer duration of treatment is thus expected to result in growth inhibition as observed in our study. This could represent effects of oestrogen deficiency or toxic effects of the drug. However, no toxic effects were observed during the study, with the only deaths being due to anaesthetic complications. Although food intake was not formally quantified, feeding behaviour and activity were similar in treated and control animals, arguing against a toxic effect of the drug. The growth-suppressing effect of aromatase

inhibition may be due to inhibition of IGF1 production due to effects of oestrogen deficiency on GH receptor expression or signalling, or loss of GH-independent hepatic production of IGF1 (Venken *et al.* 2005). Although this impaired weight gain may partially explain the observed skeletal phenotype, it would seem that even in the female model where there is weight gain, the amount of bone formation as measured by bone mineral content per weight is reduced, suggesting a direct effect of letrozole in impaired bone mass accrual in both genders (Manneras *et al.* 2007).

The third important finding of our study was the demonstration of focal prostatic hyperplasia along with increased size of the anterior and dorsal prostate lobes (when corrected for body weight) in letrozole-treated animals. As for the other novel findings, these changes were apparent well beyond the treatment period to at least the equivalent of human age of 30 years. The balance of androgens and oestrogen is critical in both prostate physiology and pathologies (Harkonen & Makela 2004, Prins *et al.* 2006, McPherson *et al.* 2008). Perturbation leading to hormonal imbalance in early neonatal life or on aging causes prostatic pathologies (Prins *et al.* 2007). The demonstration of prostatic hyperplasia due to short-term aromatase inhibition in peripubertal animals is entirely consistent with the observation that long-term oestrogen deficiency causes prostatic hyperplasia and hypertrophy in adult aromatase knockout (ArKO) mice (McPherson *et al.* 2001). These observations indicate the need for long-term follow-up for evaluation of prostatic health in young boys treated with aromatase inhibitors.

Adverse testicular effects are an important area of concern in the use of aromatase inhibitors in boys in view of impaired spermatogenesis in adult male ArKO mice (Robertson *et al.* 1999) and one man with aromatase deficiency (Carani *et al.* 1997). Although the testicular size of treated animals was increased when corrected for body weight, no obvious abnormality in testicular histology was observed in our study. Treatment with anastrozole for up to 1 year in adult male Wistar rats, however, resulted in the development of Sertoli cells-only testis in 10% of animals, with varying degrees of germ cell loss in 1–2% of seminiferous tubules (Turner *et al.* 2000). These findings suggest that severe adverse testicular effects of aromatase deficiency may manifest only after prolonged oestrogen deficiency, as seen in male ArKO mice, which are initially fertile, and impaired spermatogenesis occurs as a late event (Robertson *et al.* 1999).

The tissue effects of aromatase inhibitors in males are largely related to local oestrogen deficiency, as locally produced oestrogen plays a much more important role compared with circulating oestradiol (E_2) in males. Tissue E_2 levels are therefore more relevant than circulating E_2 levels. Studies on aromatase inhibition have failed to detect a difference in circulating E_2 levels despite evident effect on the hypothalamic–pituitary axis (Turner *et al.* 2000). E_2 levels were undetectable by the methods used in the study. Testosterone levels were not elevated in the treated animals at the end of treatment. This may be related to the fact that testosterone

levels were measured after 60 days of treatment. Importantly, Turner *et al.* in a study of effects of anastrozole in older male Wistar rats observed that although testosterone levels were higher in treated animals initially, no significant difference was observed between groups for either testosterone or FSH levels after prolonged treatment. Similarly, we were unable to demonstrate pituitary effects from assessment of FSH levels as the samples were taken only after cessation of treatment.

In contrast to observations in aromatase knockout mice (Jones *et al.* 2000) and aromatase-deficient men (Morishima *et al.* 1995) who develop an adiposity phenotype and insulin resistance, no effect of aromatase inhibition on body composition and insulin levels was observed at the end of treatment.

In summary, our study has demonstrated adverse effects of aromatase inhibition on skeleton, growth and prostate in peripubertal male rats. Although caution needs to be exercised in extrapolating these findings into the clinic due to the longer duration of aromatase inhibitor treatment in our animal model and possible species differences in drug response, the findings nevertheless point to serious potential long-term adverse effects of such therapy in peripubertal short boys. Further characterisation of long-term adverse effects of aromatase inhibitors in animal models of peripubertal males is thus highly desirable before further widespread use in young boys. Our findings also point to the need for developing selective oestrogen inhibition strategies such that oestrogen action on growth plate is inhibited, while beneficial effects in other tissues are preserved.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

The study was conducted in the laboratories of GAW, JDW and GPR. AB conceived the study. AB, GAW, VCR, JDW and GPR were involved in the planning and implementation of the study. PJS, SJM and WJA contributed to the conduct of the study. AB collected data, performed the data analysis and drafted the manuscript. All the authors reviewed and contributed to the final version of the manuscript.

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