Author/s:
McManus, JF;Nguyen, NYN;Davey, RA;MacLean, HE;Pomilio, G;McCormack, MP;Chiu, WS;Wei, AH;Zajac, JD;Curtis, DJ

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Androgens stimulate erythropoiesis through the DNA-binding activity of the androgen receptor in non-hematopoietic cells

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DR. JULIE F MCMANUS (Orcid ID : 0000-0002-0722-7828)
DR. MATTHEW P MCCORMACK (Orcid ID : 0000-0003-1536-5611)

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Abstract

Background: Androgens function through DNA and non-DNA binding-dependant signalling of the androgen receptor (AR). How androgens promote erythropoiesis is not fully understood. *Design and methods:* To identify the androgen signalling pathway, we treated male mice lacking the second zinc finger of the DNA binding domain of the AR ($AR^{\Delta ZF2}$) with non-aromatizable 5α-dihydrotestosterone (5α-DHT), or aromatizable testosterone. To distinguish direct hematopoietic and non-hematopoietic mechanisms we performed bone marrow reconstitution experiments. *Results:* In wild-type mice, 5α-DHT had greater erythroid activity than testosterone, which can be aromatized to estradiol. The erythroid response in wild-type mice following 5α-DHT treatment was associated with increased serum erythropoietin (EPO) and its downstream target erythroferrone, and hepcidin suppression. 5α-DHT had no erythroid activity in $AR^{\Delta ZF2}$ mice, proving the importance of DNA binding by the AR. Paradoxically testosterone, but not 5α-DHT, suppressed EPO levels in $AR^{\Delta ZF2}$ mice, suggesting testosterone following aromatization may oppose the erythroid-stimulating effects of androgens. Female wild-type mice reconstituted with $AR^{\Delta ZF2}$ bone marrow cells remained responsive to 5α-DHT. In contrast, $AR^{\Delta ZF2}$ mice reconstituted with female wild-type bone marrow cells showed no response to 5α-DHT. *Conclusion:* Erythroid promoting effects of androgens are mediated through DNA binding-dependent actions of the AR in non-hematopoietic cells, including stimulating EPO expression.

Keywords: genetically modified androgen receptor mouse model, androgen receptor signalling, DNA binding actions, erythropoiesis, erythropoietin, androgens, non-hematopoietic cells

Introduction

Androgens are steroid hormones responsible for male reproduction and secondary male sexual characteristics[1]. The naturally occurring androgen testosterone acts through the androgen receptor (AR), which is a ligand-dependent transcription factor[2]. Testosterone may be converted to 5α-dihydrotestosterone (5α-DHT) by 5α-reductase[3], which also acts through the AR, or aromatized to estradiol which acts via the estrogen receptor[4,5]. In classical AR signalling, upon ligand binding the AR translocates from the cytoplasm to the nucleus where it binds androgen response elements in the promoter and enhancer regions of target genes to activate or repress their transcription[6]. Non-classical AR signalling following ligand binding involves rapid activation of 2nd messenger signalling pathways, or indirect gene repression by sequestering transcription factors and is independent of DNA binding[7-9].

Androgens promote erythropoiesis in healthy[10,11] and hypogonadal men[12-14] as well as patients with anemia of chronic kidney disease[15,16]. Conversely, blockade of androgens used for the treatment of men with prostate cancer results in anemia[17-20]. How androgens stimulate erythropoiesis remains ill-defined with both direct and indirect mechanisms proposed. Androgens may increase levels of erythropoietin (EPO)[21] or directly stimulate erythroid progenitors[22-24]. Androgens may also stimulate iron-dependent erythropoiesis by suppression of hepcidin via inhibition of BMP6/SMAD signalling[21] or activation of EGF/EGFR signalling in the liver[25,26].

We have developed a genetically modified androgen receptor mouse model where we targeted exon 3 resulting in an in-frame deletion of the 2nd zinc finger of the DNA-binding domain of the AR (AR$^{\Delta ZF2}$). Although the first zinc finger remains intact, the DNA-binding dependent actions are abolished, whilst the ligand-binding dependent actions remain functional[27]. Using this unique

$AR^{\Delta ZF2}$ mouse model, we show that androgens promote erythropoiesis through the DNA binding-dependent actions of the AR in non-hematopoietic cells, which includes stimulation of EPO. These findings may have implications for the use of androgens to stimulate erythropoiesis in the treatment of anemia.

Materials and methods

Mice

$AR^{\Delta ZF2}$ mice lack a functional DNA binding domain. This was achieved by an in-frame deletion of exon 3 which encodes the second zinc finger of the DNA binding domain of the AR, leaving the first zinc finger intact. The $AR^{\Delta ZF2}$ mouse line was created by breeding exon 3 floxed AR mice with CMV-Cre mice to generate $AR^{WT/\Delta ZF2}$ heterozygous females, and maintained on a congenic C57BL/6J background as described previously[27,28]. The male $AR^{\Delta ZF2}$ mice and their wild-type male and females litter mates used in this study were generated by crossing $AR^{WT/\Delta ZF2}$ heterozygous female mice with wild-type C57BL/6J male mice.

Mice were housed in the Austin Health animal facility or AMREP (Alfred Medical Research and Education Precinct) PAC facility, Alfred Health, with a 12-hour light/dark cycle and food and water supplied *ad libitum*. All procedures involving animals were pre-approved by the Austin Health or AMREP animal ethics committees, A2005/02113 and E/1259/2012/M respectively.

Androgen treatment

Wild-type male and female mice, and male $AR^{\Delta ZF2}$ mice were administered androgens (Sigma-Aldrich, Australia) for 48 hours, 1, 2 or 6 weeks at 8-13, 7, 7 or 4 weeks of age respectively by subcutaneous dorsal surgical insertion of three 1.4cm Silastic tubing implants with either vehicle (empty), or containing 5α-DHT or testosterone (approximately 11mg per implant)[29]. Such implants deliver sustainable supraphysiological levels of androgens for up to 10 weeks[29]. For details of androgen treatments and endpoints refer to Table S1 in the Supporting Online Content.

Re-population studies

Adult wild-type female and male $AR^{\Delta ZF2}$ mice (aged between 7 and 16 weeks) were irradiated with 2 doses of 550 Rads at an interval of three hours and then transplanted with 10^7 bone marrow cells via the tail vein. Following a recovery period of 8 weeks, reconstituted mice had subcutaneous dorsal surgical insertion of either vehicle or 5α-DHT implants (as described above) for a period of 2 weeks.

Tissue harvest

Blood was collected from the tail vein into potassium EDTA tubes (Sarstedt, Numbrecht, Germany) for hematology testing and into tubes without anticoagulant for serum EPO measurement. Bone marrow was flushed from the femora using 5% fetal calf serum in phosphate buffered saline and a single cell suspension prepared by drawing the bone marrow back and forth three times through a 21G needle. A single cell suspension of spleen cells was prepared using a 70μm sieve. Liver, kidney and spleen were frozen at -80^{o}C for subsequent gene expression analyses.

Hematology

Blood was collected from either cardiac puncture post-euthanasia or via tail vein. Red cell indices were measured using the Advia 2120 (Siemens) or Hemavet (Dres Scientific) automated blood analyser. For reticulocyte analysis on peripheral red blood cells, 1μl aliquots of blood were incubated in 0.5mL thiazole orange (100ng/mL, Sigma) and 0.2mL Ter119-PE (BD Pharmingen, catalogue number 553673) for 30 min at room temperature protected from light then analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Serum EPO was quantified using the R&D Systems Quantikine ELISA kit (Catalogue number MEP00B) according to manufacturer's instructions.

Real-time quantitative PCR

Total RNA was isolated from mouse kidneys, liver, bone marrow and spleen using Trizol (Thermofisher) according to manufacturer's instructions with or

without further clean-up using the Qiagen RNeasy kit. cDNA was synthesised from total RNA using random hexamer priming (Promega) and Superscript III (Invitrogen).

Inventoried TaqMan gene expression assays (Applied Biosystems) were used to quantitate gene expression levels of *Epo* (Mm01202755_m1), *Hamp* (gene encoding Hepcidin) (Mm04231240_s1) and *Fam132b* (gene encoding Erythroferrone) (Mm00557748_m1), and housekeeping genes *Abl* (Mm00802029_m1) and *Hprt* (Mm01545399_m1).

Gene expression of *Epo* and *Hamp* was normalised to *Hprt,* and *Fam132b* to *Abl,* a commonly used housekeeping gene for RTqPCR in bone marrow and spleen. Gene expression levels were determined by the delta delta Ct method using the same wild-type male mouse treated with vehicle as the reference sample.

Statistical analysis

Comparisons between two groups were analysed using two-tailed unpaired Student's t-test with Holm-Šídák correction for multiple comparisons using GraphPad Prism Version 8.

Results

Steady state hematopoiesis in male AR$^{\Delta ZF2}$ mice

To determine the significance of classical DNA binding-dependent actions of the AR on erythropoiesis, we examined baseline red cell indices in male AR$^{\Delta ZF2}$ mice. Baseline hemoglobin, red cell count and hematocrit did not differ between adult male AR$^{\Delta ZF2}$ mice and wild-type male or female littermates (Table 1), consistent with observations in a liver-specific complete androgen receptor knockout model that deletes both classical and non-classical AR function[30]. In contrast to normal erythroid parameters, neutrophils were decreased and lymphocytes increased in male AR$^{\Delta ZF2}$ mice as previously observed[31,32].

Effects of androgen treatment on erythropoiesis

To examine the effects of androgens on erythropoiesis, we treated mice with two endogenous ligands, either testosterone, which can act through both the

AR, and the estrogen receptor following aromatization, or the non-aromatizable 5α-DHT, which acts only through the AR. To confirm effective administration of androgens, we demonstrated increased mass of androgen-responsive tissues (kidney, seminal vesicles) and decreased testis mass, in wild-type but not male $AR^{\Delta ZF2}$ mice (Figure 1).

Increases in red cell indices in wild-type mice were detected in initial studies with androgen treatment duration of 6 weeks and 2 weeks. Reticulocyte numbers (data not shown) and EPO levels (2 week data not shown) were not increased at these time points. Since the erythropoietic response to androgens in the 2 week treatment group was greater than in the 6 week group, we introduced a one week treatment group to identify any increase in reticulocytes and/or serum EPO. Increased reticulocytes were observed in wild-type mice at this time point but there was no change in serum EPO (data not shown). Since reticulocytes have been shown to increase approximately one week post administration of recombinant EPO[33], we included an earlier time point, 48 hours, to detect any potential rise in serum EPO.

Wild-type mice (male and female) treated with 5α-DHT showed a significant increase in erythropoiesis that was most apparent after 2 weeks (Figure 2B-2D). In contrast, 5α-DHT had no effect on erythropoiesis in male $AR^{\Delta ZF2}$ mice (Figure 2B-2D). The stimulatory effects of androgens in wild-type mice were associated with an increase in reticulocytes after the first week of treatment (Figure 2A). These effects were less apparent after 6 weeks of treatment, especially with testosterone (Figure 2E). Given the lower potency of testosterone, we didn't include a two week treatment with testosterone for this study. The weaker erythropoietic response to testosterone may reflect its lower potency compared with 5α-DHT[2] or its estradiol effect through aromatization[5]. Therefore, our results have demonstrated that the classical DNA binding-dependent actions of the AR are required to increase erythropoiesis.

Androgens increase serum erythropoietin levels and iron bioavailability

We examined the effect of androgens on serum EPO as a potential mechanism of action. After 48 hours treatment with 5α-DHT or testosterone, serum EPO in

both male and female wild-type mice was increased 2-4 fold (Figure 3A). In contrast to wild-type mice, 5α-DHT or testosterone or treatment had no effect on serum EPO levels in male $AR^{\Delta ZF2}$ mice (Figure 3A). The increase in serum EPO was reflected with increased kidney *Epo* expression in wild-type male and female mice but not male $AR^{\Delta ZF2}$ mice in response to DHT, however did not reach statistical significance (p=0.06) (Figure 3B). Consistent with the non-sustained effect of androgens on red cell indices, serum EPO levels normalised in mice treated with 5α-DHT for 6 weeks (Figure 3C). In contrast to 5α-DHT, testosterone had less stimulatory effect on serum EPO at 48 hours in WT/F mice (Figure 3A) and paradoxically, suppressed serum EPO in wild-type male mice treated for 6 weeks (Figure 3C). This suppression of serum EPO by testosterone was also observed in male $AR^{\Delta ZF2}$ mice, indicating it occurs independently of AR DNA binding. Overall, these results show that androgens transiently increase serum EPO via the DNA binding activity of the AR, and aromatizable androgens have a prolonged effect to decrease serum EPO via a non DNA binding AR-mediated action.

Erythroferrone is produced by erythroblasts in response to stimulation by EPO and acts to increase the available iron for hemoglobin synthesis. Consistent with increased serum EPO, bone marrow and spleen erythroferrone mRNA (*Fam132b*) levels increased in response to 5α-DHT in female wild-type mice but not male $AR^{\Delta ZF2}$ mice (Figure 3D and 3E). The lack of response in male wild-type mice to 5α-DHT is not unexpected since cells in male mice are accustomed to an environment with high androgen levels. Given the reported repressive effects of androgens on hepcidin (*Hamp*)[21], we examined the effect of 5α-DHT on liver *Hamp* gene expression in wild-type male and female mice and male $AR^{\Delta ZF2}$ mice. In the steady state (vehicle-treated mice), gene expression of *Hamp* was two-fold higher in female wild-type mice compared with male wild-type mice (Figure 3F) consistent with previous reports[34,35].

Hamp gene expression in male $AR^{\Delta ZF2}$ mice was comparable to female wild-type mice, consistent with AR-dependent suppression of *Hamp* (Figure 3F). Furthermore, 5α-DHT or testosterone treatment significantly suppressed *Hamp*

gene expression in female wild-type mice which have very low baseline androgen levels. Together, these results show that androgens increase serum EPO (Figure 3A), which in turn enhances iron delivery for erythropoiesis through promoting erythroferrone expression and suppression of hepcidin.

Androgens promote erythropoiesis by a non-hematopoietic mechanism

It has been proposed that androgens stimulate erythropoiesis by direct binding to the AR in erythroid progenitors, however, the presence of the AR in hematopoietic cells has not been definitively proven[36]. To determine if androgens promote erythropoiesis through direct binding of the AR in erythroid progenitors, we generated male $AR^{\Delta ZF2}$/wild-type female bone marrow chimeras. We chose cross gender male/female reciprocal donors/recipients for two reasons: firstly because male $AR^{\Delta ZF2}$ mice have complete androgen insensitivity and develop more like females, and secondly to maximise the potential effects of exogenous androgen treatment, since wild-type female cells come from a low-androgen exposure environment, while wild-type male cells are conditioned to high-androgen concentrations. Male $AR^{\Delta ZF2}$ mice repopulated with bone marrow cells from wild-type female mice showed no increase in hematocrit or reticulocytes following treatment with 5α-DHT (Figure 4). In contrast, wild-type female mice repopulated with bone marrow cells from male $AR^{\Delta ZF2}$ mice responded to 5α-DHT with increased hematocrit (Figure 4A). The average reticulocyte percentage was also increased in this group of mice however did not reach statistical significance ($p=0.06$) (Figure 4B). Thus, we conclude that androgens promote erythropoiesis through binding to the AR with a functionally intact DNA-binding domain in non-hematopoietic cells.

Discussion

In this study, we investigated the mechanisms of androgen mediated erythropoiesis using a genetically modified androgen receptor mouse model ($AR^{\Delta ZF2}$) in which the DNA binding-dependent actions of the AR are abolished. The rationale for this model was based on patients with complete androgen insensitivity syndrome (CAIS) where the second zinc finger of the AR is

deleted[37]. Deletion of the AR in CAIS does not perturb erythropoiesis (personal communication),[38].

Using male $AR^{\Delta ZF2}$ mice we show that stimulation of erythropoiesis by the AR is DNA binding-dependent (Figure 2). We further show that androgens stimulate EPO production, which in turn promotes iron utilisation through increased erythroferrone and suppression of hepcidin (Figure 3). Finally, we generated bone marrow chimeras to show that the erythropoietic effect of androgens is through DNA binding of the AR in non-hematopoietic cells rather than in erythroid progenitors (Figure 4).

Our results conflict with *in vitro* studies which suggested direct action of androgens on erythropoietic precursors[22-24]. However, our findings are consistent with immunohistochemistry findings where the AR was not detectable in erythropoietic precursors[36]. Stimulation of EPO has been a proposed mechanism of androgens. Here, we show that this stimulation of EPO occurs via DNA binding-dependent actions of the AR. We also show that DNA binding by the AR is required for androgens to promote AR repression of hepcidin gene expression, which may also enhance erythropoiesis by increasing iron availability. Other mediators of androgen action on erythropoiesis must also be present, as a recent study showed that androgens can stimulate erythropoiesis in hepcidin knockout mice[30].

We found that as expected, the less potent androgen testosterone was less effective than 5α-DHT at stimulating erythropoiesis. Indeed, testosterone suppressed *Epo* expression in male $AR^{\Delta ZF2}$ mice, demonstrating opposing modulatory effects of testosterone and 5α-DHT. The suppression of *Epo* expression may be attributable to aromatization of testosterone to estradiol and its subsequent activation of the estrogen receptor[39] or alternatively through non-classical signalling pathways of the AR[28]. In future experiments with the use of an aromatase inhibitor we propose to further investigate the possibility that the estrogen receptor is opposing the action of the AR.

The transient rise in EPO and the reduced effects on hematocrit following 6 weeks of androgen treatment suggests a compensatory change or tolerance to elevated levels of androgens. A transient rise in EPO has previously been

described in female wild-type mice[21] and in healthy older men, where EPO levels were increased for up to 3 months but normalised by 6 months[40]. A comparable erythropoietic response pattern was described in the same cohort of healthy older men where elevated hematocrits returned to normal on a background of supraphysiological testosterone levels, suggesting development of androgen tolerance when present for extended periods[40].

In summary, this work shows that androgens, especially the non-aromatizable endogenous ligand 5α-DHT, stimulate EPO production and consequently erythropoiesis through the DNA binding-dependent signalling pathway of the AR in non-hematopoietic cells. Understanding why this effect is short-lived may further enhance the therapeutic potential of androgens for promoting erythropoiesis.

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	Wild-type male (n=6-12)	$AR^{\Delta ZF2}$ male (n=10-14)	Wild-type female (n=8-15)
Hemoglobin (g/dL)	13.68 ± 1.87	13.55 ± 1.5	13.44 ± 1.03
Red cells (x 10^{12}/L)	8.92 ± 0.13	8.93 ± 0.09	8.83 ± 0.08
Hematocrit (%)	51.17 ± 0.55	50.34 ± 0.76	49.87 ± 0.62
MCV (fl)	57.67 ± 0.84	55.6 ± 0.98	55.5 ± 0.94
MCH (pg)	15.33 ± 0.21	15.2 ± 0.13	15.13 ± 0.23
Reticulocytes (%)	3.60 ± 0.99	3.67 ± 0.97	2.42 ± 0.67
Neutrophils (x10^{9}/L)	1.29 ± 0.25	0.69 ± 0.12*	0.74 ± 0.11
Lymphocytes (x10^{9}/L)	5.07 ± 0.68	7.56 ± 0.89*	5.87 ± 1.00

Table 1. Full blood examination of genetically modified male androgen receptor mouse ($AR^{\Delta ZF2}$). Blood parameters obtained from adult mice. Number of mice in parentheses. Mean ± SEM are shown. Two-tailed students t-test compared with wild-type male mice. *p <0.05 vs wild-type male. Abbreviations: MCV, Mean cell volume; MCH, mean cellular haemoglobin.

Figure Legends

Figure 1

Effects of 5α-dihydrotestosterone (DHT) or Testosterone treatment for 6 weeks in wild-type male (WT/M) and female mice (WT/F), and male $AR^{\Delta ZF2}$ mice ($AR^{\Delta ZF2}$/M) on the mass of androgen responsive tissues: (A) kidney, (B) testis and (C) seminal vesicles. Data are expressed as mean ± SEM, *P* values represent treatment with DHT or testosterone vs. vehicle; ns, not significant. The sample number per treatment group: n = 5-14 mice

Figure 2

The relative effects of 5α-dihydrotestosterone (DHT) and Testosterone on red cell indices: (A) Treatment of wild-type male (WT/M) and wild-type female (WT/F) mice with DHT for one week increased absolute reticulocyte numbers. Treatment of wild-type male and wild-type female mice with DHT for two weeks increased (B) Red cell count, (C) Hemoglobin, and (D) Hematocrit. Red cell indices in male $AR^{\Delta ZF2}$ ($AR^{\Delta ZF2}$/M) mice were unchanged. (E) Treatment with DHT but not testosterone for six weeks increased hematocrit in wild-type male mice. Data are expressed as mean ± SEM, *P* values represent treatment with DHT vs. vehicle (A to D) or treatment with DHT vs. vehicle or testosterone (E); ns, not significant. The sample number per treatment group: n = 4-13 mice

Figure 3

Effects of 5α-dihydrotestosterone (DHT) or Testosterone treatment on erythropoietic growth factors: (A) Treatment with DHT or testosterone for 48 hours increased serum EPO in wild-type male (WT/M) and female (WT/F) mice but not male $AR^{\Delta ZF2}$ mice ($AR^{\Delta ZF2}$/M) (B) Treatment with DHT for 48 hours increased kidney *Epo* expression in wild-type male (p=0.06), and wild-type female (p=0.06) but not male $AR^{\Delta ZF2}$ mice. (C) Treatment with testosterone for 6 weeks decreased serum EPO in both wild-type male and male $AR^{\Delta ZF2}$ mice compared with vehicle. Treatment with DHT or testosterone for 48 hours increased bone marrow *Fam132b* (D) and spleen *Fam132b* (E) in wild-type

female but not male $AR^{\Delta ZF2}$ mice. (F) Treatment with DHT for 48 hours decreased liver *Hamp* in wild-type female but not male $AR^{\Delta ZF2}$ mice. Data are expressed as mean ± SEM, *P* values represent treatment with DHT vs. vehicle or testosterone (A to F) and testosterone vs. vehicle (C); ns, not significant. The sample number per treatment group: n = 3-12.

Figure 4

Mouse repopulation studies demonstrate that AR targets are erythroid cell extrinsic. Effects of DHT treatment on red cell indices in chimeric mice: (A) Treatment for 2 weeks increased Hematocrit in wild-type female (WT/F) mice transplanted with donor bone marrow from male $AR^{\Delta ZF2}$ mice ($AR^{\Delta ZF2}$/M) and (B) Treatment for one week increased reticulocytes in wild-type female mice transplanted with donor bone marrow from male $AR^{\Delta ZF2}$ mice (p=0.06). Data are expressed as mean ± SEM, *P* values represent DHT vs. vehicle; ns, not significant. The sample number per treatment group: n = 3-8.

	Wild-type male (n=6-12)	$AR^{\Delta ZF2}$ male (n=10-14)	Wild-type female (n=8-15)
Hemoglobin (g/dL)	13.68 ± 1.87	13.55 ± 1.5	13.44 ± 1.03
Red cells (x 10^{12}/L)	8.92 ± 0.13	8.93 ± 0.09	8.83 ± 0.08
Hematocrit (%)	51.17 ± 0.55	50.34 ± 0.76	49.87 ± 0.62
MCV (fl)	57.67 ± 0.84	55.6 ± 0.98	55.5 ± 0.94
MCH (pg)	15.33 ± 0.21	15.2 ± 0.13	15.13 ± 0.23
Reticulocytes (%)	3.60 ± 0.99	3.67 ± 0.97	2.42 ± 0.67
Neutrophils (x10^{9}/L)	1.29 ± 0.25	0.69 ± 0.12*	0.74 ± 0.11
Lymphocytes (x10^{9}/L)	5.07 ± 0.68	7.56 ± 0.89*	5.87 ± 1.00

Figure 1

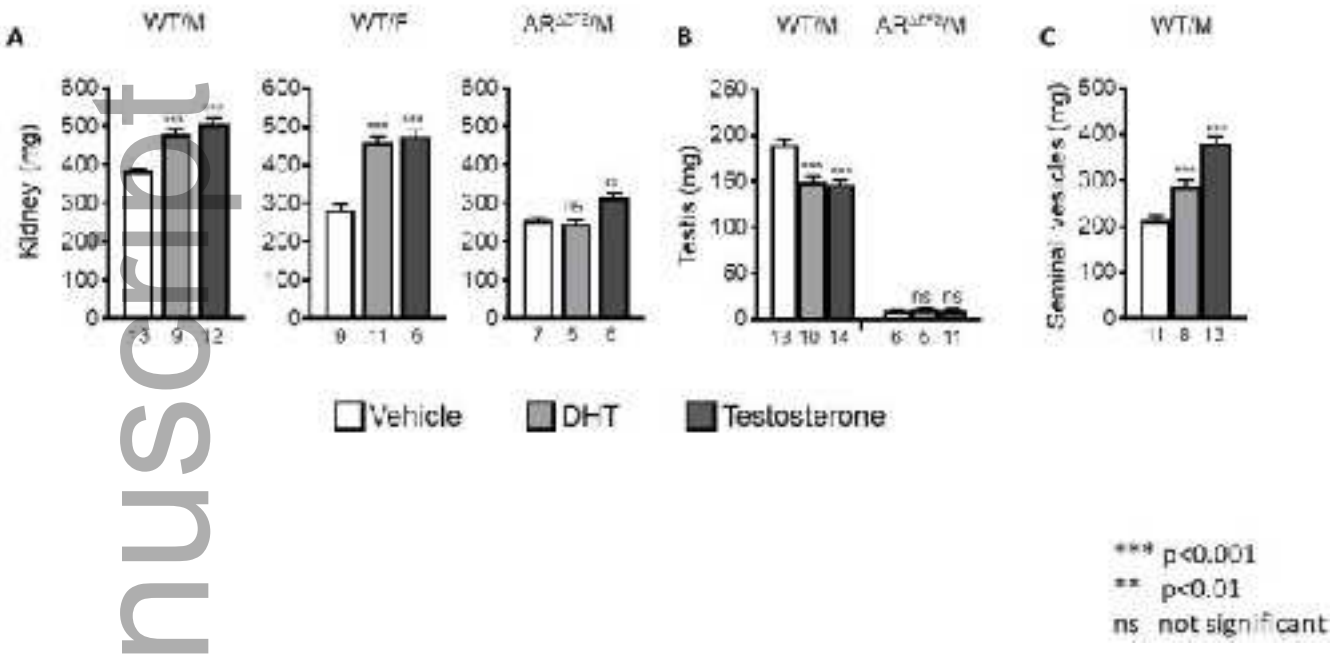

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Figure 2

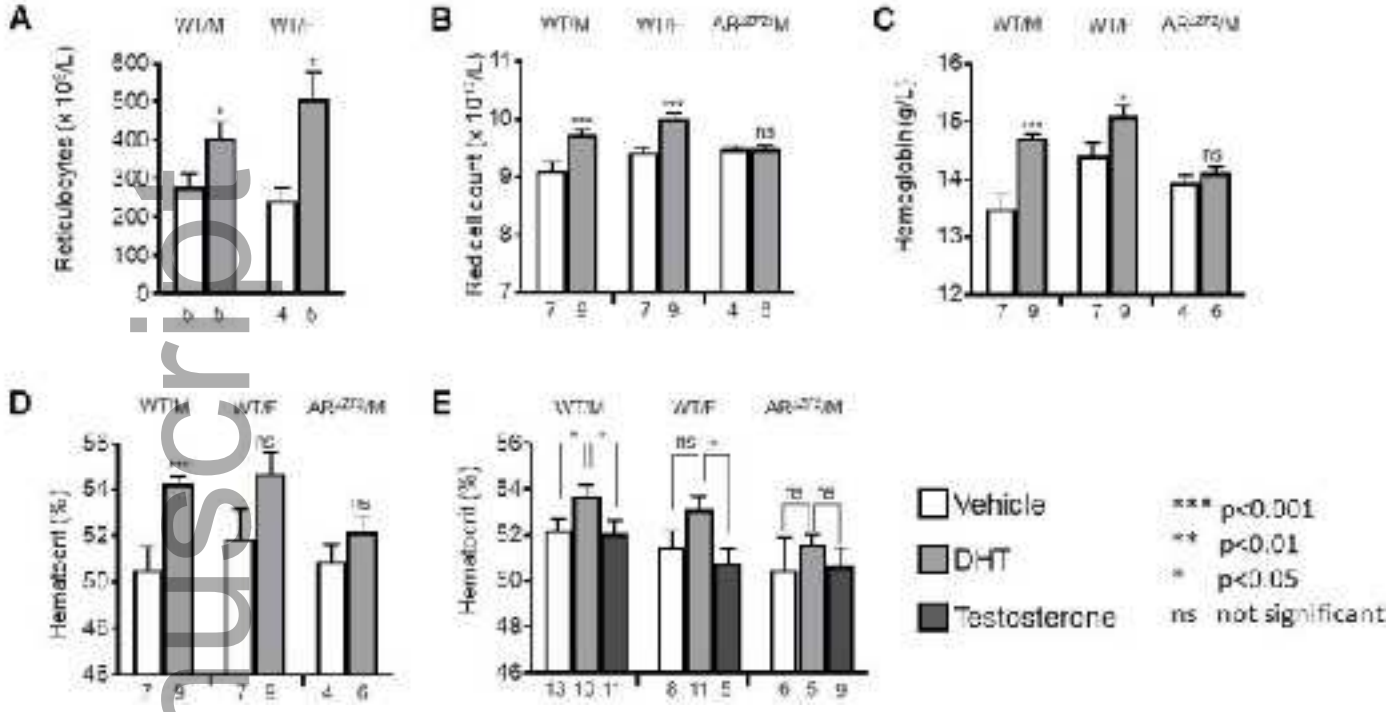

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Figure 3

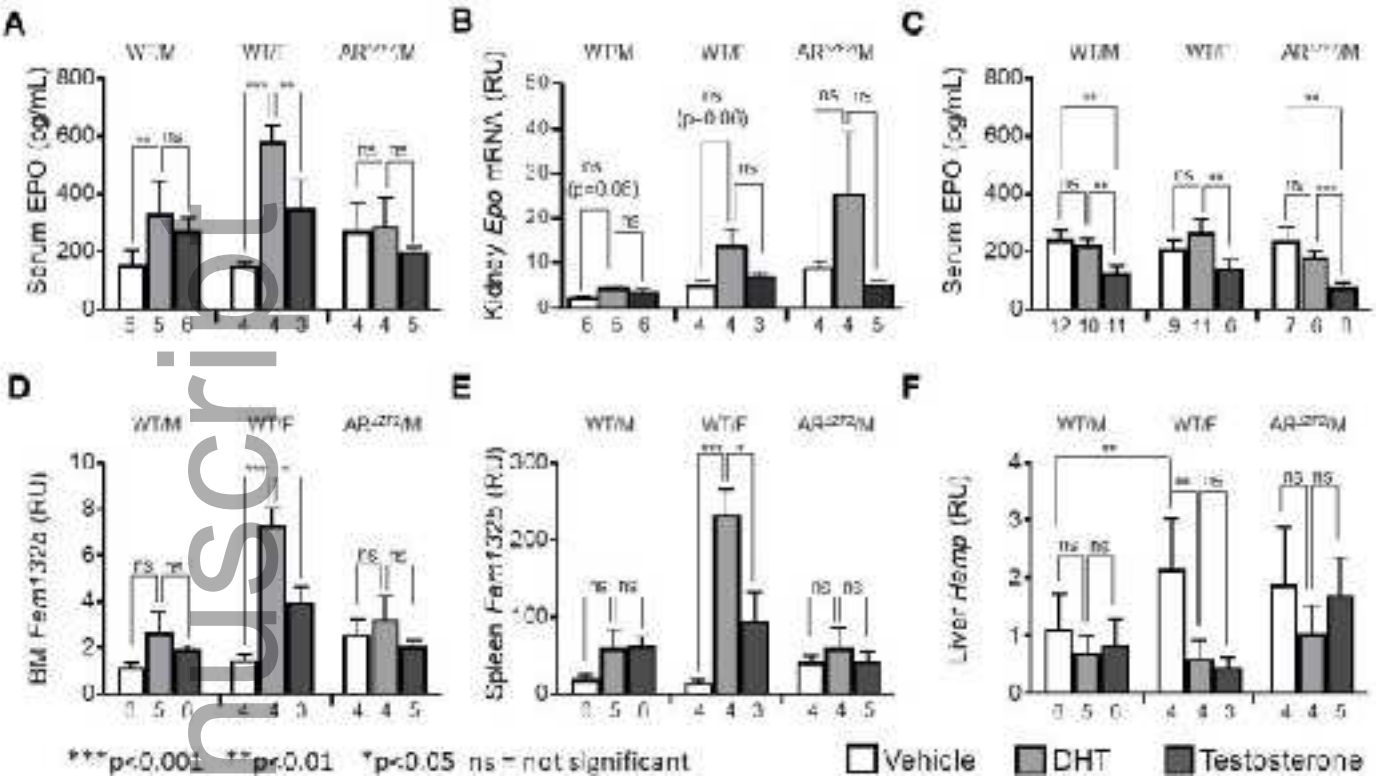

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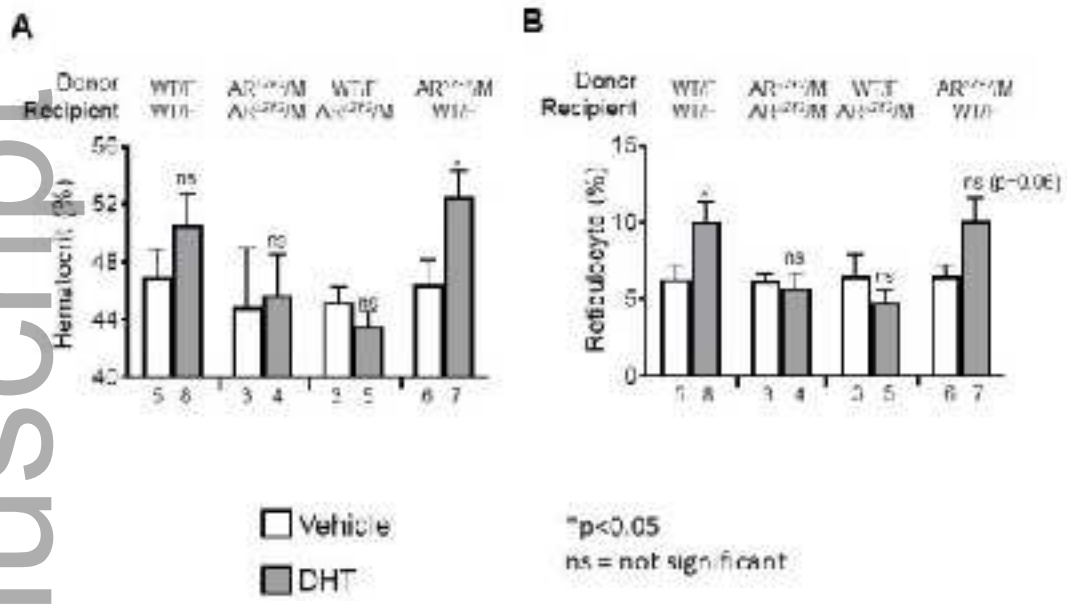
Figure 4



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