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14. ABSTRACT – Endocrine disrupting chemicals (EDCs) are present in our everyday life and many of them act to oppose androgens including the fungicide, Vinclozolin. Most of the studies on EDCs are conducted in rodents and have limited utility when extrapolating the findings to humans. In order to test the concept that Vinclozolin alters human prostate development and induces disease, we used our model system to study human prostate development and maturation over 8-12 weeks, comparable to the process that takes decades in men. Briefly we use rodent stroma to create a niche in which human stem cells are directed to become human prostatic epithelia. Task 1 sought to test if the rodent stromal niche (obtained after exposure to Vinclozolin) was altered so that differentiation and development to normal human prostate tissue was aberrant leading to a disease phenotype. Surprisingly, our results showed development of the prostatic phenotype was completely blocked and was not predicted. These novel findings are fundamental to understanding how normal prostatic differentiation proceeds and our publications show the essential role of stroma is perturbed by Vinclozolin, define the prostatic phenotype induced by Vinclozolin and demonstrate the involvement of NFkB signalling pathways.					
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INTRODUCTION

Endocrine disrupting chemicals (EDCs) are present in our everyday life and many of them act to oppose androgens including the fungicide, Vinclozolin. Most of the studies on EDCs are conducted in rodents and have limited utility when extrapolating the findings to humans. In order to test the concept that Vinclozolin alters human prostate development and induces disease, we used our model system to study human prostate development and maturation over 8-12 weeks, comparable to the process that takes decades in men. Briefly we use rodent stroma to create a niche in which human stem cells are directed to become human prostatic epithelia. Task 1 sought to test if the rodent stromal niche (obtained after exposure to Vinclozolin) was altered so that differentiation and development to normal human prostate tissue was aberrant leading to a disease phenotype. Surprisingly, our results showed development of the prostatic phenotype was completely blocked and was not predicted. These novel findings are fundamental to understanding how normal prostatic differentiation proceeds and our publications show the essential role of stroma is perturbed by Vinclozolin, define the prostatic phenotype induced by Vinclozolin and demonstrate the involvement of NFkB signalling pathways.

BODY

Research Outcomes associated with Tasks as outlined in the approved Statement of Work.

Aim:

The aim of the experiments described in Task 1, was to expose pregnant female rats to vinclozolin and test if the inductive and instructive properties of the prostate stroma is altered and disrupts differentiation of human prostate tissue derived from hESC.

Methods

Briefly, this involved the assignment of pregnant dams to one of four treatment groups: 100, 200 or 400 mg Vinclozolin/Kg/Day (in 2.5µl of corn oil/g body wt) or corn oil vehicle control. Dams transiently exposed by oral gavage from gestational days (GD) 14 to 19, and left to litter down. Vinclozolin exposure to male pups was confirmed by measuring anogenital distance (1).

Tissue recombinants generated from rodent derived prostate mesenchyme from male pups in the litters and combined with human embryonic stem cells. Tissues harvested for analysis after 12 weeks of growth and evidence of Vinclozolin treatment assessed using the following parameters:

Tissue size: Graft volume was determined by stereological method.

Histopathology: Tissue sections selected from the entire block stained for H&E and examined by microscopy. Evidence for specific lesions was examined to identify:

PIN lesions: Nucleolus size and nucleoli prominence determined as evidence of PIN lesions.

Focal PIN lesions identified by up-regulation of AR, ER α and p63 and the down regulation of E-cadherin as previously described (2).

Inflammation: Areas of inflammation identified based on accumulation of neutrophils and lymphocytes and lymphoid aggregates in the stroma. (2).

Epithelial hyperplasia: Specific morphological criteria assessed (3) and accurately estimated using CAST software. Counting frames and systematic uniform random sampling methods were adapted from those used to estimate epithelial morphology in gut (4). Epithelial 'branches' were counted for a minimum of 6 sections taken through each graft. Counts were expressed per unit of section area, with the mean for each graft used for comparison.

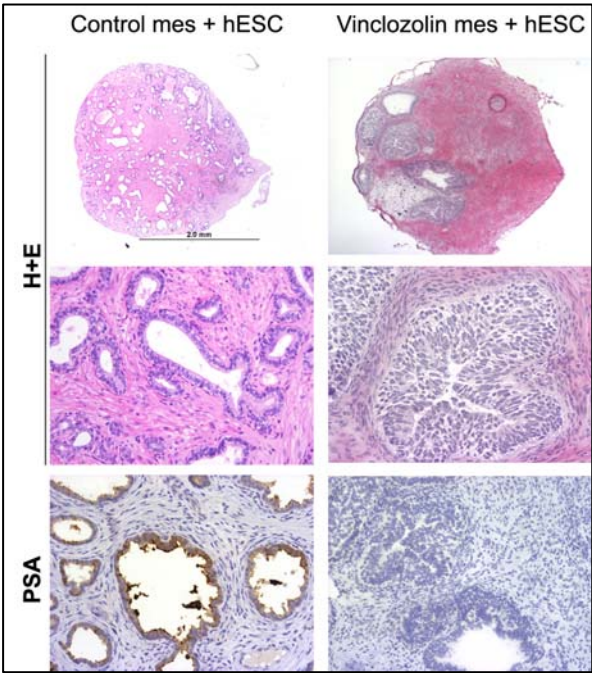
Altered secretory epithelial cell activity and reduced PSA: Image analysis was performed to estimate conversion to PSA expressing glandular tissue as previously described (5). Entire grafts were sectioned and beginning from a randomly selected tissue section, a systematic

uniform sampling procedure applied to select 10% of the tissue throughout the explant. Using CAST software, area that was glandular/PSA positive was measured and expressed as a percentage of the total section area. The mean for each graft was derived from the mean values of at least 10 sections. The mean value of glandular tissue (PSA positive) for the graft type was obtained from not less than 4 grafts per group.

Results:

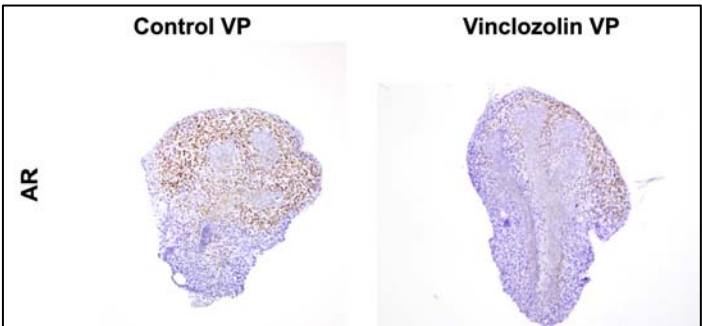
Perturbations in normal human prostate differentiation in Vinclozolin + hESC recombinants

Analysis of tissue recombinants at 12 weeks revealed the perturbation of normal human prostate tissue development and maturation using Vinclozolin exposed mesenchyme. The observed effect was not as predicted and showed fundamental failure to allow normal prostate development and maturation (Figure 1). The absence of PSA expressing glandular tissue in Vinclozolin + hESC recombinants confirmed the absence of formation of human prostate tissue (Figure 1).



Aberrant androgen receptor expression in Vinclozolin exposed mesenchyme

The inductive and instructive potential of the mesenchyme and importance of mesenchymal androgen signaling in



normal prostate branching and development has been well established. Analysis of Vinclozolin exposed mesenchyme revealed a significant down regulation in androgen receptor (AR) expression (Figure 2).

Thus, the perturbation of normal prostatic differentiation in Vinclozolin + hESC recombinants is due to the reduced AR expression and consequential aberrant androgen signaling.

Outcomes and conclusions:

We predicted one of 2 outcomes. The predicted outcome was that Vinclozolin would perturb human prostate development or maturation and reduce graft size, reduce epithelial differentiation and secretory activity and may even have caused PIN lesions. This outcome was partly achieved, in that there was a failure of human prostatic graft development, attributable to the reduced AR expression. We know AR expression is crucial for prostate development and this would explain why the tissue failed to develop. However this outcome did not allow us to determine if there were any PIN lesions or evidence of inflammatory pathologies.

Task 2:

Aims and approach

The original aim was to determine if the effects of Vinclozolin were transgenerational and evident in the F2 generation, but the outcome of Task 1 required us to evaluate the nature of the failure of human prostate development from the tissue recombinants. Specifically this led us to determine the aberrant effects of *in utero* Vinclozolin exposure on the rodent prostate to identify the similarity to human prostatic inflammation or prostatitis.

Results.

Our data demonstrate rats transiently exposed to Vinclozolin develop prostatitis immediately post-puberty. At 2 months (8weeks) of age; the incidence of prostatitis in these relatively young animals was 100%. The early onset of prostatitis, is comparable to the onset of non-bacterial early-onset prostatitis in younger men, and implicates the EDC, Vinclozolin, as a causative factor in the etiology of prostatitis.

Specifically we report key observations:

Post-pubertal prostatitis occurs in all of the Vinclozolin treated rats.

The incidence of prostatic inflammation or prostatitis was 100% in post-pubertal rats treated in utero with Vinclozolin.

Table 1: Incidence of Prostatitis in post-pubertal F1 males	
Group	Incidence
Control	0/17
Vinclozolin	24/24

The prostates showed significant pathology compared to controls. Prominent, but focal, regions of inflammation were observed with an increase in the proportion of inflammatory cells, particularly leukocytes and including macrophages (ED1) surrounding the ducts and vasculature – see figure 3.

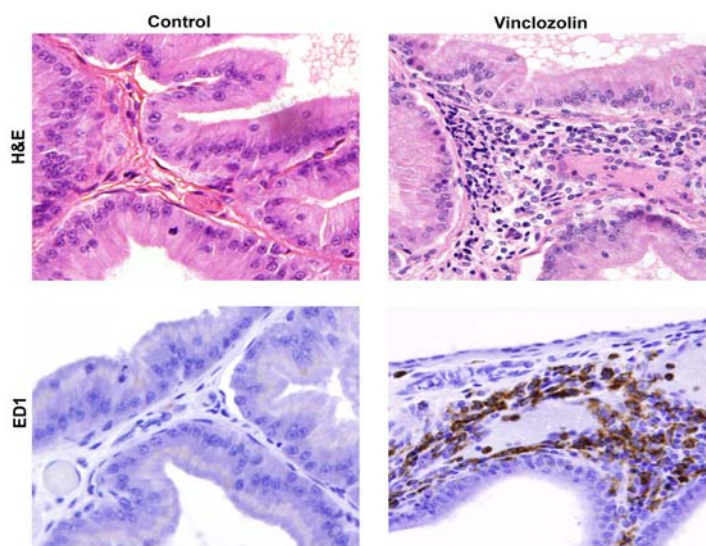


Figure 3 The left hand panels show control prostate tissues with normal histology (H&E) and no macrophage infiltration as assessed by immunostaining for ED1. The top right hand panel is an H&E section of Vinclozolin treated prostate tissue showing evidence of inflammation. The bottom right

hand panel shows brown staining representing localisation of a ED1 antibody and evidence of infiltration of macrophages.

NFkB signalling pathway is implicated in the onset of prostatitis in post-pubertal rat tissue

A number of genes activated and significantly up-regulated - Table 2- several of these genes are

associated with NFkB signalling.

To determine if NFkB itself was

upregulated, we examined if

there was nuclear localisation of

phosphorylated NFkB which

only occurs upon activation.

Table 2: Genes up regulated in Vinclozolin vs control tissues	
Fold-increase	Genes
>5	Interleukin 1; Interleukin 6 receptor α ; Interleukin 8 receptor α , macrophage migration inhibitory factor;CD40 ligand
>10	Interleukin 6 signal transducer; Interleukin-17 precursor; Nitric oxide synthase 2; Toll-like receptors 1 and 4-6; Tumor necrosis factor (ligand) superfamily member 4
>20	Transforming growth factor beta 1; Toll-like receptors 2, 3 and 9; Tumor necrosis factor superfamily member 2; Tumor necrosis factor receptor superfamily member 1a & 1b

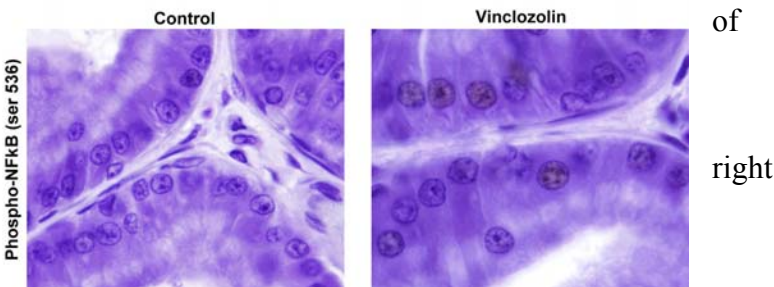
Figure 4: left panel shows no evidence

phosphor-NFkB (Ser536) in control

prostate tissue. In contrast, Figure 4:

panel shows evidence of nuclear NFkB

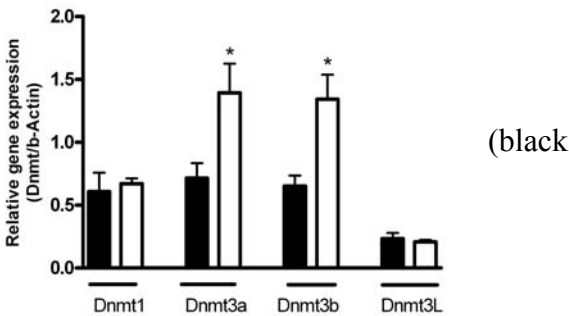
(arrows) demonstrating activation of NFkB.



Deregulation of epigenetic associated dimethyltransferase (DNMT) enzymes occurs with prostatitis in post-pubertal rat tissue

Figure 5 shows the relative levels of DNA

methyltransferases in prostatic tissues from control



bars) and EDC treated (open bars) postpubertal rats. Significant increases (* $p < 0.05$) observed in DNMT3a and 3b, indicative of epigenetic modification by DNA methylation due to EDC.

KEY RESEARCH ACCOMPLISHMENTS

Bulleted list of key research accomplishments emanating from this research.

- Demonstration that Vinclozolin disrupts human prostate development in tissue recombinants composed of human ESCs and rodent stroma, implicating this EDC as a potential hazard to human prostate health.
- Demonstration that Vinclozolin causes prostatitis, implicating this EDC as a causative factor in prostatitis, a condition known to increase the risk of prostate cancer.

REPORTABLE OUTCOMES

Provide a list of reportable outcomes that have resulted from this research to include:

Manuscripts – Refer to Appendix 1 for full text

Cowin PA, Foster P, Pedersen J, Hedwards S, McPherson S, Risbridger GP (2008) Early onset endocrine disruptor induced prostatitis in rodents (Accepted February 2008; Environmental Health Perspectives) Impact Factor: 5.86 (*In Press*)

Abstracts of Presentations – Refer to Appendix 2 for full text

1. **Cowin PA**, Foster P, McPherson SJ, Risbridger GP (2007) Prostate disease begins in the womb: Early origins of prostatic inflammation caused by transient exposure to the endocrine disrupting chemical Vinclozolin. Southern Health Research Week, Clayton, Victoria Australia (**Poster presentation**)

2. **Cowin PA**, Foster P, McPherson SJ, Risbridger GP (2007) Prostate disease begins in the womb: Early origins of prostatic inflammation caused by transient exposure to the endocrine disrupting chemical (EDCs) – Vinclozolin. Lorne Cancer Conference. Lorne, Victoria Australia (**Poster presentation**)
3. **Cowin PA**, Foster P, McPherson SJ, Risbridger GP (2007) Prostatic Inflammatory Atrophy induced following transient *in utero* exposure to the endocrine disruptor Vinclozolin Healthy Start for a Healthy Life: The Wintour's Tale, A Satellite Conference of DOHaD. Melbourne, Australia (**Oral Presentation**)
4. **Cowin PA**, Foster P, McPherson SJ, Risbridger GP (2007) Transient in utero exposure to the endocrine disruptor Vinclozolin induces inflammation and atrophy in the post- but not pre-pubertal prostate. 50th Annual Scientific Meeting, Endocrine Society of Australia, Christchurch, New Zealand. (**Oral Presentation**)
5. Risbridger GP, **Cowin PA**, McPherson SJ, Foster P (2007) Transient endocrine disruption induces prostate pathologies upon aging. 50th Annual Scientific Meeting, Endocrine Society of Australia, Christchurch, New Zealand. (**Seminar presentation GPR**)

Degrees obtained that are supported by this award

- ❖ To be included in doctoral thesis submitted by Ms Prue Cowin PhD (June 2008)

Funding applied for based on work supported by this award

Based on these data, application was made for Australian government funding from NH&MRC for a project grant.

CONCLUSION:

The role of endocrine disrupting chemicals (EDCs) in the early origins of adult prostate disease is of concern and controversy to the lay and scientific communities. These data are the first to unequivocally implicate the anti-androgenic activity of EDCs as causative factors in the aetiology of prostatitis in the rat, providing novel insight to the origins of this disease.

The majority (>90%) of prostatitis cases are ascribed to unknown (non-bacterial) origins and the symptoms, both acute and chronic, are common, bothersome and burdensome in terms of health-related quality-of-life (6, 7). The economic impact of prostatitis includes an estimated annual expenditure in the US of >\$84 million for diagnosis and management, excluding subsequent pharmaceutical costs (8-10). As there are extensive gaps in our understanding of prostatitis aetiology, many of these current expenditures may be ineffective and a waste of resources. **These data are the first to unequivocally implicate EDCs as a causative factor and fill an important knowledge gap on the aetiology of prostatitis.**

Overall, the robust incidence of inflammation in 100% of young adult rats mimics more closely human non-bacterial prostatitis that occurs in young men. Ninety percent of prostatitis cases are of unknown cause and these data are the first to implicate anti-androgenic EDCs as a causative factor in the aetiology of this inflammatory disease of the prostate via activation of the classical NF κ B inflammatory pathway. Whilst the level of Vinclozolin utilised in this study far exceeds that observed in the environment and projected human exposure, this study raises further concerns that *in utero* exposures to EDCs, with anti-androgenic activity have long range effects that include the development of prostatitis in early adult life, and provide further impetus to test the efficacy of treatments that block or abrogate NF κ B signalling in the treatment of prostatitis.

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List all references pertinent to the report using a standard journal format (i.e. format used in *Science*, *Military Medicine*, etc.).

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APPENDICES

Appendix 1: Manuscript (in Press) in Environmental Health Perspectives

Early Onset Endocrine Disruptor Induced Prostatitis in the Rat

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Running Title: Endocrine Disruptor Induced Prostatitis

Key Words: Anti-androgen, Endocrine Disruptors, Inflammation, Prostate, Prostatitis, Vinclozolin

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Funding: This research was funded by the US Army Department of Defence, Prostate Cancer Research Program Exploration-Hypothesis Development Award (W81XWH-07-1-0126) (GPR) and supported [in part] by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences (PF).

Abbreviations:

AGD	Anogenital distance
AP	Anterior prostate
AR	Androgen receptor
BPH	Benign prostate hyperplasia
cDNA	Complementary deoxyribonucleic acid
DP	Dorsal prostate
EDC	Endocrine disruptor
E	Estrogen
FgF10	Fibroblast Growth Factor 10

GD	Gestational day
LP	Lateral prostate
NF κ B	Nuclear factor kappa B
PCa	Prostate cancer
PIN	Prostatic intra-epithelial neoplasia
PIA	Proliferative inflammatory atrophy
PND	Post-natal day; prostate
SV	Seminal vesical
T	Testosterone
VP	Ventral

Outline of section headers:

Abstract

Introduction

Material and Methods

Animals

Treatment

Necropsy of male littermates

Hormone Analysis

Tissue Collection

Tissue Separation

Histology

mRNA Extraction

Oligo GEArray

Stereology

Wholemount Immunolabelling

Analysis of Branching Morphogenesis

Statistical Analysis

Results

Compound purification data

Post-natal day 0 litter data

Gross analysis of male off-spring following in utero treatment

Absence of perturbations in branching morphogenesis in neo-natal off-spring

Normal prostate development until puberty with early onset of prostate inflammation in post-pubertal off-spring

Epithelial aberrations in post-pubertal off-spring without evidence of pre-malignancy

Discussion and Conclusions

List of References

Tables

Table 1: Effects of 6 day *in utero* administration of Vinclozolin at PND0

Table 2: Effects of 6 day *in utero* administration of Vinclozolin on PND28 and PND56 reproductive organ weights

Table 3: Effects of 6 day *in utero* administration of Vinclozolin on key NFκB dependant inflammatory genes at post-natal day 56 compared to Control treatment

Figure Legends

Figures

Figure 1: Absence of perturbations in branching morphogenesis in neo-natal off-spring exposed *in utero* to Vinclozolin.

Figure 2: Serum Testosterone.

Figure 3: Early onset post-pubertal prostatitis in animals exposed *in utero* to Vinclozolin.

Figure 4: Epithelial attenuation in post-pubertal animals following *in utero* Vinclozolin treatment.

Abstract

Background: Androgens are critical for specifying prostate development with the fetal prostate sensitive to altered hormone levels and endocrine disrupting chemicals (EDCs) that exhibit estrogenic or anti-androgenic properties. Prostatic Inflammation (prostatitis) affects 9% of men of all ages and >90% of cases are of unknown aetiology. **Objectives:** This study aimed to evaluate effects of *in utero* exposure to the anti-androgenic EDC Vinclozolin during the period of male reproductive tract development, on neo-natal, pre- and post-pubertal prostate gland function of male off-spring. **Methods:** Fetal rats were exposed to Vinclozolin (100mg/kg/bw) or vehicle control (2.5ml/kg/bw) *in utero* from gestational day 14 to 19 via oral administration to pregnant dams. Male off-spring were aged to 0, 4 or 8 weeks before tissue analysis. **Results:** *In utero* exposure to Vinclozolin was insufficient to perturb prostatic development and branching, although androgen receptor and mesenchymal fibroblast growth factor 10 expressions were down-regulated. Prostate histology remained normal until puberty but 100% of animals displayed prostatitis post pubertally (56 days). Prostatic inflammation was associated phosphorylation and nuclear translocation of NFκB and post-pubertal activation of pro-inflammatory NFκB dependant genes including the chemokine Interleukin-8 and cytokine Transforming Growth Factor β1. Significantly, inflammation arising from Vinclozolin exposure was not associated with the emergence of pre-malignant lesions, such as prostatic intra-epithelial neoplasia (PIN) or proliferative inflammatory atrophy (PIA) and hence mimics non-bacterial early-onset prostatitis that commonly occurs in young men. **Conclusions:** These data are the first to unequivocally implicate EDCs as a causative factor and fill an important knowledge gap on the aetiology of prostatitis.

Introduction

Environmental pollutants or industrial chemicals disrupt, and have the potential to alter, the action of gonadal steroid hormones by virtue of their anti-androgenic or estrogenic properties and in doing so, effect hormonal balance (11-13). Reproductive tract development during fetal and neonatal life is hormonally regulated and in an undifferentiated state, lacking compensatory homeostatic mechanisms to prevent adverse effects of endocrine disrupting chemicals (EDCs) (14, 15). Thus, the organisational effects of EDCs on the developing reproductive tract can be permanent and irreversible.

Dissimilar to prostate cancer (PCa) and benign prostate hyperplasia (BPH) that predominantly affect ageing men, prostate inflammation (prostatitis) affects 9% of men of all ages (10). The majority (>90%) of prostatitis cases are ascribed to unknown (non-bacterial) origins and the symptoms, both acute and chronic, are common, bothersome and burdensome in terms of health-related quality-of-life (6, 7). The economic impact of prostatitis includes an estimated annual expenditure in the US of >\$84 million for diagnosis and management, excluding subsequent pharmaceutical costs (8-10). As there are extensive gaps in our understanding of prostatitis aetiology, many of these current expenditures may be ineffective and a waste of resources. Thus, it is imperative we better understand this disease, one that has received relatively little attention compared to BPH and PCa.

Whilst increased levels of developmental or environmental estrogens have been linked to the increased incidence of prostate disease (16, 17), chemicals with anti-androgenic activity are potentially of greater importance because androgens are critical to establishing the male phenotype. Vinclozolin (3-(3,5-dichlorophenyl)-5-methyl-oxazolidine-2,4-dione) is an anti-androgenic systemic dicarboximide fungicide used widely throughout Europe and the United States in the control of diseases caused by *Botrytis cinerea*, *Sclerotinia sclerotiorum*, and *Monilinia spp.* Vinclozolin is degraded to several

metabolites: 2-[[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenic acid (M1) and 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2), which are competitive antagonists of androgen receptor (AR)-ligand binding, rather than 5 α -reductase enzyme inhibitors (18, 19). Vinclozolin has a half-life of 23 days when sprayed as Ronilan (a 50% mixture of Vinclozolin) on soil, with previous reports showing Vinclozolin exposure induces malformations such as cryptorchidism, hypospadias and Leydig cell hyperplasia, and permanent changes in sexually dimorphic structures, such as anogenital distance (AGD) and areola/nipple retention (20). These effects occur before hypothalamic-pituitary-gonadal axis formation and long after Vinclozolin has been cleared from the pup, thus are organisational effects rather than due to interruption of a feedback loop via the pituitary.

Recent interest in Vinclozolin arose from reports that transient embryonic exposure during embryonic gonadal sex determination (gestational days (GD) 8-14) appears to alter the male germ line epigenome and subsequently promotes transgenerational adult onset disease, including testis and immune abnormalities, prostate and kidney disease and tumour development (21). A preliminary report stated prostate disease, including inflammation and epithelial atrophy, occurs in aged rats (12-14 months), although the incidence of prostatic lesions across four generations of male rats was only 10% (22). These findings are interesting (although the low incidence of prostatic lesions is not compelling) and at the same time controversial because of the EDC purity and timing and route of its administration *in utero*.

Vinclozolin, when purchased commercially, requires purification and recrystallisation to obtain >99% purity and ensure any effects are not due to contaminants. Human exposure to Vinclozolin occurs by oral ingestion enabling metabolism to the more potent AR antagonists (M1 and M2). Direct

intraperitoneal administration runs the risk of producing effects not observed by the conventional oral route, such as uterine irritation and changes in uterine blood flow. The timing of Vinclozolin exposure also varies the effect on male reproductive tract development. A window of sensitivity for prostate development occurs when ARs are activated between GD 14-19, rather than during embryonic gonadal sex determination around GD8-14 (23). Commonly, the outcomes of any transient *in utero* treatments are examined in ageing animals. However, anti-androgen effects also manifest at other times including pre- and post-puberty, when hormone action is critical for normal prostate maturation and function. Altogether, these variations in treatment protocol may account for the low incidence of prostatic lesions reported by Anway et al who used intraperitoneally administered unpurified Vinclozolin, during GD8-14 and did not study outcomes until 12-14 months of age (21).

Therefore, the aim of this study was to evaluate effects of fetal exposure to purified Vinclozolin, administered orally to pregnant dams during the period of male reproductive tract development (GD 14-19), on pre- and post-pubertal prostate gland function in male off-spring.

Material and Methods

Animals

All animal procedures were conducted according to National Health and Medical Research Council (NHMRC) guidelines and animal experimentation ethics committee at Monash Medical Centre, Clayton, Australia (MMCA/2006/22). Animals were treated humanely and with regard for alleviation of suffering. Time mated female outbred Sprague-Dawley rats were obtained from Monash University Central Animal Services (Clayton, Australia) on gestational day (GD) 8 and housed at Monash Medical Centre Animal House (Clayton, Australia) under controlled 12 hour light-dark cycle and temperature conditions. Animals were fed *ad libitum*. GD 0 was the day plugs were observed in the vagina of mated females. Dams and offspring were housed together until weaning (post-natal day (PND) 21) when male litter mates were group-housed, ≤ 4 per cage. Dams and female offspring were euthanized humanely by CO₂ asphyxiation and not subjected to post mortem examination.

Treatment

The treatment regime was performed as previously described (23). On GD14 dams were weighed and animal allocation to treatment groups done by body weight (bw) randomization to ensure unbiased weight distribution among groups. Dams were assigned 1 of 2 treatment groups (0, 100mg.kg.bw Vinclozolin) (n=16 dams per group) and 1 of 3 time points of collection (PND 0, PND 28 or PND 56) (n=8 dams at PND 0; n=4 dams at PND 28 and PND 56 per treatment group). Dams were orally dosed using a micropipette daily at 1000hr from GD14 to 19 with 100mg/kg/bw body weight Vinclozolin (Research Triangle Park, NC, USA) or corn oil vehicle Control (Sigma; 2.5 ml/kg/bw) and examined for clinical signs of toxicity. The dose chosen corresponds to a level commonly used to investigate Vinclozolin effects on male reproductive tract formation, inducing an array of male reproductive tract

malformations at high incidence without maternal toxicity. As the pubertal period in the rodent is controversial, puberty has been defined in relation to functional puberty or the time at which sperm appear and serum Testosterone rises. This occurs around post-natal day 43 in rats (24, 25).

Necropsy of male littermates

Male offspring were collected at PND 0, 28 or 56, weighed, euthanized by decapitation (PND 0) or CO₂ asphyxiation (PND 28 and 56) and blood collected by cardiac puncture for hormonal analysis. External genitalia, including scrotum, prepuce and penis were visually examined and anogenital distance (AGD) measured with a calliper.

Hormone Analysis

Serum testosterone (T) levels were measured by ANZAC Research Institute (Sydney, Australia) as previously described (26).

Tissue Collection

Using a dissecting microscope (SZX12, Olympus Corporation, Tokyo, Japan) and dissecting tools ventral (VP), dorsal (DP), lateral (LP) and anterior (AP) prostate lobes, testes and seminal vesicles (SV) were dissected from PND 28 and 56 animals and wet weights recorded. For isolation of PND 0 prostates, urogenital tracts were removed and VPs micro-dissected in a modified watch glass (Maximov depression slide; San Francisco, CA), in the presence of dissecting media (basal medium of Dulbecco's Modified Eagles Media (DMEM) and Hams F-12 (1:1 vol/vol) supplemented with

penicillin and streptomycin (5mls/ltr) and fungizone (20µg/ml) at pH 7.3). Pair matched organs were fixed in Bouin's fixative or immediately frozen in liquid nitrogen and stored at -80°C.

Tissue Separation

PND 0 VP's for epithelial and mesenchymal RNA analysis were digested in 1% trypsin (Difco, Detroit, MI) in Hank's calcium and magnesium free Balanced Salt Solution (HBSS; Gibco, Invitrogen, Vic, Australia) for 60 minutes. Mesenchyme and epithelia were mechanically separated, immediately frozen in liquid nitrogen and stored at -80°C.

Histology

Fixed tissues were dehydrated, processed and embedded in paraffin. Serial 5µm sections were cut and mounted onto Superfrost Plus+ coated slides (Menzel-Glaser®, Germany). Tissue sections were stained with Harris' haematoxylin and eosin (H&E) or used for immuno-histochemistry.

Immunohistochemistry was performed using the DAKO Autostainer Universal Staining System (DAKO A/S, Denmark) (27). Antibodies were purchased from Santa Cruz Biotechnology Inc.(CA, USA) to: AR (AR (N-20)), TGFβ1 (SC-146), TLR4 and Fgf10 (H-121:SC_7917); PCNA (clone PC10) DAKO Corporation, (Denmark); CD68 (ED1) Sapphire Bioscience Pty Ltd (NSW, Australia); phospho-NFκB p65 (Ser536) Cell Signaling Technology Inc. (MA, USA). Antibodies were used as previously described (28-30) or according to company specifications.

mRNA Extraction

Total RNA was extracted from prostate tissues using TRIzol Reagent (Invitrogen Life Technologies, Rockville, MD) according to manufacturer specifications and as previously described (27).

Oligo GEArray

Gene expression analysis was carried out using GEArray™ DNA Microarray (PND56: array no. ERN-011.2, PND0: EMM-014) (SuperArray Bioscience Corporation, MD, USA) according to manufacture's directions. Analysis was conducted on a minimum of 4 samples per group in duplicate. Briefly, complementary deoxyribonucleic acid (cDNA) was synthesised from pure RNA using the TrueLabelling-AMP™ 2.0 kit (SuperArray Bioscience Corporation, MD, USA). cDNA was amplified followed by a 24 hour complimentary RNA (cRNA) synthesis reaction. cRNA concentration and purity was determined by UV Spectrophotometry. Following generation and purification of cRNA, array hybridisation was performed using the Oligo GEArray® HybPlate Basic Protocol (SuperArray Bioscience Corporation, MD, USA) according to manufactures directions. Briefly, arrays were subjected to pre-hybridisation before hybridization with the labelled target cRNA and incubated for 24 hours at 60°C, then washed repeatedly in stringency washes. Detection was performed using the Chemiluminescent Detection Kit (SuperArray Bioscience Corporation, MD, USA). Briefly, arrays were incubated at room temperature in dilute AP-Streptavidin for 10 minutes, rinsed in buffer and incubated with a chemiluminescent detection chemical, CDP-Star®. Images were acquired immediately using X-ray exposure. X-ray images were captured using a scanner and saved as 16 bit TIFF images. Data analysis was completed using GEArray Expression Analysis Suite (SuperArray Bioscience Corporation, MD, USA), with expression normalised to a specific set of house-keeping genes.

Stereology

An unbiased estimate of the terminally differentiated secretory epithelial cell population and incidence of inflammation was obtained using stereological techniques, based on the Cavalieri principle (31) and as previously described in the testes and prostate (32-34). Stereological analysis was performed using a BX-51 microscope (Olympus Corp.) and a JVC TK-C1380 video camera (Victor Company of Japan Ltd, Japan) coupled to an IBM computer. Images were projected directly onto a video screen and utilising the CAST V1.10 software (Computer Assisted Stereological Toolbox) (Olympus Danmark A/S, Denmark.) tissue sections were mapped at x 40 magnification to define tissue boundaries. Beginning from a random point, sampling was conducted at predetermined intervals along x- and y- axes using a three-by-three point grid counting frame. A minimum of 10 sections per animal uniformly spaced throughout explants and 5 animals per group were utilised. To accurately differentiate terminally differentiated secretory epithelial cells, tissue sections stained for CKHMW were utilized, with positively and negatively stained CKHMW cells identified and percentages of positive and negative cells was determined. To determine the incidence of inflammatory lesions random fields were designated as positive or negative for inflammation. Abnormal inflammatory regions were classified as areas that displayed chronic inflammation as represented in Figure 3D.

Wholemout Immunolabelling

Branching morphogenesis analysis was conducted on PND 0 VPs. Individual VPs were placed in methanol and stored at -20°C. Immunolabelling was performed as previously described (35). Briefly, tissues were permeabilized in 0.2% (v/v) Triton X-100 (Sigma, St Louis, USA) and 5µg/ml Sodium Borohydride in PBS (pH 8.0) for 15 mins. Non-specific binding was blocked with 'Superblock' blocking buffer (Pierce, IL, USA) for 1 hour at room temperature, before overnight incubation at 4 °C

with a monoclonal mouse anti-human CKHMW (Dako Corporation, USA) (5 μ g/ml immunoglobulin G (IgG). Tissues were incubated with 5 μ g/ml goat anti-mouse IgG secondary antibody (F[ab]₂ fragments) conjugated with fluorescein isothiocyanate (FITC) (Dako Corporation, USA) for 90 min at room temperature. VPs were mounted on slides using Vectorshield fluorescent mounting medium (Vector, CA, USA) with coverslips mounted on nail polish platforms, to maintain 3D patterns.

Analysis of Branching Morphogenesis

Serial optical images of CKHMW stained wholemount tissues were generated at 2 μ m interframe steps using an Olympus confocal microscope, captured, and stored in 8 bit BMP format using Fluroview software (Olympus) as previously described (36). Confocal images were subsequently used to construct a 3D skeleton representing the original ductal pattern of the gland, using lines running through the centre of each ductal/epithelial branch. A full description of this process was previously reported for the study of branching morphogenesis in kidney (37, 38) and prostate (36). The resultant algorithm provides fully automated measurements of the branch length (in pixels and micrometres) and cumulative surface areas (in square pixels) of the individual ducts as they appear in all the frames. Total ductal length was calculated by adding together individual branch lengths from multiple ducts within a lobe. The value of the surface areas was multiplied by 1.5522 (pixel area), to convert it into micrometres, then by 2 (μ m, interframe steps) to obtain total volume (in μ m³) of individual ducts. Numbers of branch points, branches, and terminal tips were automatically generated by the software.

Statistical Analysis

All pup data was analysed individually and nested by dam to yield litter means. To test for significance of treatment effects litter was corrected for as a main effect variable using one-way analysis of covariance (ANCOVA) on SPSS (v.16 SPSS Inc., Chicago, USA) and data expressed as litter mean $\pm$ standard error of the mean (SEM). AGD and organ weights were analyzed with body weight as a covariate. Control and Vinclozolin were compared using an F test, with the significance threshold employed at a level of 5% ($p < 0.05$). Analysis of Control and Vinclozolin stereological data was performed using a two-tailed paired t test, and using Prism 4.0 software (GraphPad Software, Inc., San Diego, CA).

Results

Compound purification data

Vinclozolin was obtained from BASF AG (Research Triangle Park, NC, USA) as Ronilan ® EG (a 50% Vinclozolin mixture) and purified and recrystallised. Catalogued as lot no. 357-141A, it was certified as being of >99% purity by ChemService Inc (West Chester, PA, USA).

Post-natal day 0 litter data

Gestating Sprague Dawley rats were transiently exposed to Vinclozolin (100 mg.kg.day) and compared to corn oil vehicle treated control (2.5ml.kg.day) from gestational day (GD) 14-19. Vinclozolin did not induce maternal toxicity or affect normal pregnancy as no dams presented dystocia or delivered late. Dam weight gain through the dosing period was not significantly different between groups (Table 1). Live litter size and sex ratio were not affected by Vinclozolin treatment, with sex confirmed at puberty (Table 1).

Gross analysis of male off-spring following in utero treatment

Male off-spring exposed *in utero* were analysed at post-natal day (PND) 0 (day of birth), PND 28 (pre-pubertal) or PND 56 (post-pubertal) for any weight differences or gross morphological abnormalities. Pup weights at PND 0 were significantly reduced between *in utero* Control and Vinclozolin treated groups ($p < 0.05$) (Table 1), however no significant changes to body weight were demonstrated at PND 28 or 56 ($p < 0.05$; Table 2). Anogenital distance (AGD) is a sensitive indicator of anti-androgenicity, and AGD was significantly reduced at all ages following *in utero* Vinclozolin exposure compared to Control (Table 1 and 2). Covariate analysis demonstrated the PND 0 AGD reductions were not due pup weight reductions. At PND 28, compared to Control, *in utero* Vinclozolin treatment did not significantly reduce testis, seminal vesicles (SV), ventral (VP), anterior (AP), dorsal (DP) and lateral (LP) prostate weights ($p < 0.05$) (Table 2). Analysis of external genitalia revealed undescended testis in $35.93 \pm 4.37\%$ of pre-pubertal animals exposed *in utero* to Vinclozolin, compared with none in Control. At PND 56, *in utero* Vinclozolin treatment significantly reduced SV and VP weights and significantly increased AP weight ($p < 0.05$) (Table 2). No significant differences in LP, DP and testis weights were observed (Table 2). Malformations of external genitalia included cleft prepuce, incomplete preputial separation, cleft phallus and hypospadias and observed in $47.02 \pm 7.92\%$ of post-pubertal animals exposed *in utero* to Vinclozolin compared to none in Control. Analysis of serum testosterone (T) levels revealed no significant differences between *in utero* treatment with Control and Vinclozolin at any post-natal age (Figure 1).

Whilst all prostate lobes were collected and analysed, only the ventral prostate (VP) is reported herein as in addition to being the most commonly reported lobe with respect to EDC exposures, it is the most

androgen sensitive lobe (39, 40) and it was predicted the actions of an anti-androgenic chemical would be more likely to induce effects in this lobe.

Absence of perturbations in branching morphogenesis in neo-natal off-spring

The inductive and instructive potential of the mesenchyme and importance of mesenchymal androgen signalling in normal prostate branching and development has been well established. To examine whether *in utero* Vinclozolin exposure perturbed normal mesenchymal signalling in offspring, gene array analysis of 113 common growth factors was performed on neonatal mesenchyme and revealed significant (>1.5 fold) down regulation of several mesenchymal genes including fibroblast growth factor (*Fgf10*) in prostates from animals exposed *in utero* to Vinclozolin compared to Control and confirmed by immuno-protein localisation (data not shown). Since estrogen exposure down regulates *Fgf10* and perturbs normal prostate ductal branching (41), we investigated whether the anti androgen-induced reduction in mesenchymal *Fgf10* was associated with developmental abnormalities. Using a computer-based method that allows temporal and spatial alterations in branching morphogenesis as a result of experimental manipulations to be examined branching morphogenesis in neonatal tissues was analysed. Despite mesenchymal *Fgf10* reductions, prostate size was normal with no significant differences present in ductal number, length, volume or branch points in neonatal specimens between prostates from animals exposed *in utero* to Vinclozolin and Control (Figure 2).

Normal prostate development until puberty with early onset of prostate inflammation in post-pubertal off-spring

No gross morphological differences between *in utero* Vinclozolin and Control treatment were observed in PND28 (pre-pubertal) prostates (Figure 3A, B). However, analysis of prostate specimens at PND56 (post-pubertal) revealed the onset of prostate inflammation in *in utero* Vinclozolin exposed males

(Figure 3C, D). Prominent, but focal, regions of inflammation were observed in 100% of animals, with an increase in the proportion of inflammatory cells, particularly leukocytes and macrophages, surrounding the ducts and infiltrating into the vessels (Figure 3D). Increased macrophage infiltration was evident in prostates from animals exposed *in utero* to Vinclozolin, as demonstrated by immuno-localisation of ED1 (Figure 3E, F), although this was absent in control animals. *In utero* Vinclozolin treatment resulted in a significant increase ($p < 0.05$) in the percentage of prostatic inflammatory lesions, identified by stereological analysis from $1.4 \pm 0.80\%$ to $16.8 \pm 3.72\%$ (Figure 3G).

Pro-inflammatory stimuli and immune responses are commonly controlled by the nuclear factor-kappa B (NF κ B) family of transcription factors. In unstimulated cells, NF κ B is sequestered in the cytoplasm of cells and activated when phosphorylated and translocated to the nucleus. Activation of NF κ B in prostates exposed *in utero* to Vinclozolin was confirmed by nuclear immuno-protein localisation of phospho-NF κ B p65 (Ser536) antibody which detects NF κ B p65 only when phosphorylated at serine 536. In prostates exposed to vehicle control few immuno-positive cells were identified (Figure 3H, I).

Activation of NF κ B subsequently induces transcription of many NF κ B dependent genes, including those encoding inflammatory cytokines and chemokines. To determine the distinct pattern of gene expression following activation of NF κ B signalling by Vinclozolin treatment, pathway specific gene array analysis of 113 key genes involved in the inflammatory response was performed. A significant (>1.5 fold) up- and down-regulation of 69 and 34 genes respectively was observed in prostate tissues of PND 56 animals exposed *in utero* to Vinclozolin compared with animals exposed *in utero* to the vehicle Control (Table 3). These data show increased activation of classic pro-inflammatory NF κ B dependant genes including chemokines such as Interleukins (IL)-1 α , IL-6 and IL-8) and cytokines such

as Transforming Growth Factor (TGF)- β and Tumour Necrosis Factor (TNF)- α , as well as other ligands and receptors including toll-like receptors (TLR) 1-6 and 9 and TNF receptors.

Several key NF κ B dependant genes were selected to examine transcriptional activity by immuno-protein localisation, including TLR-4 and TGF- β 1. Heightened expression of TLR-4, an important innate immune receptor, was confirmed by increased immuno-protein localisation, particularly in the stromal and peri-ductal compartments of tissues exposed *in utero* to Vinclozolin (Figure 3J, K). A significant up-regulation of TGF- β 1 expression was observed in tissues exposed *in utero* to Vinclozolin (Figure 3L, M) correlating with a significant down regulation of the immuno-suppressive cytokine and TGF- β 1 negative regulator IL-10 (Table 3).

Epithelial aberrations in post-pubertal off-spring without evidence of pre-malignancy

In addition to the 100% penetrance of prostatic inflammation observed in PND56 prostate specimens, *in utero* exposure to Vinclozolin also induced focal epithelial attenuation (reduction in epithelial cell height and thinning of ductal structure) in all animals. A reduction in epithelial AR was observed concurrently with a reduction in terminally differentiated secretory epithelia (Figure 4A, B). Loss of terminally differentiated secretory epithelia and epithelial attenuation was shown by immuno-localisation of the basal cell marker, CKHMW (Figure 4C, D), which showed a continuous layer of basal cells compared to a discontinuous layer observed in Control, and confirmed by stereological analysis (Figure 4E).

Prostatic inflammation associated with atrophy and proliferation has been reported as a pre-malignant lesion in men, known as proliferative inflammatory atrophy (PIA). Although PIA has not been confirmed in rodents, proliferative activity was examined by immuno-localisation of proliferating cell nuclear antigen (PCNA) (Figure 4F, G). In epithelial attenuated glands of tissues exposed *in utero* to Vinclozolin there was an apparent loss of proliferative activity with a reduction in immuno-positive epithelial cells, demonstrating the absence of pathology comparable to PIA in these tissues. There was no evidence of other prostatic lesions, such as pre-malignant prostatic intraepithelial neoplastic (PIN) lesions.

Discussion and Conclusions

The role of endocrine disrupting chemicals (EDCs) in the early origins of adult prostate disease is of concern and controversy to the lay and scientific communities. These data are the first to unequivocally implicate the anti-androgenic activity of EDCs as causative factors in the aetiology of prostatitis in the rat, providing novel insight to the origins of this disease in which >90% of human cases are of unknown cause (10).

The longer term consequences of *in utero* Vinclozolin exposure include the development of gross malformations of the male reproductive tract such as the epididymis, vas deferens, seminal vesicles, prostate, external genitalia (hypospadias), cryptorchidism and testicular injury and permanent change in sexually dimorphic structures (23). A reduction in nuclear epithelial AR in prostate tissues from animals exposed *in utero* to Vinclozolin correlates with previous studies demonstrating rapid AR degradation following anti-androgen binding (19).

The immediate effects of reduced AR and mesenchymal *Fgf10* expression in tissues exposed *in utero* to Vinclozolin do not result in any perturbations in prostate branching. This implies that other androgen-regulated paracrine factors produced by the mesenchyme were sufficient to compensate and induce normal differentiation and development. Furthermore, the absence of a branching effect following *in utero* treatment during primary gland genesis may indicate that *Fgf10* may only act with concurrent chemical exposure in the neonate when secondary branching morphogenesis occurs. These findings contrast effects reported following neonatal estrogen exposure, in which reductions in mesenchymal *Fgf10* inhibits branching morphogenesis (41).

Prostatic inflammation is a common feature of endocrine disruption by estrogenic and anti-androgenic chemicals (14, 42, 43). Our results demonstrate the absence of any morphological changes prior to puberty but an inflammatory response in all young post-pubertal (56 day old) prostates following *in utero* anti-androgenic exposure. Activation of the NFκB inflammatory pathway was evident with a significant down regulation of AR expression. There is considerable evidence to show cross talk between AR and NFκB (44) thus it is reasonable to postulate that the persistent repression of AR signalling induced by Vinclozolin results in androgenic activity that is insufficient to suppress NFκB signalling pathways, resulting in inappropriate activation of NFκB and the emergence of prostatitis. Whilst the exact mechanism by which estradiol exposure promotes an inflammatory response in the adult prostate has not yet been determined, the anti-androgen induced inflammation is associated with activation of the ‘canonical’ pro-inflammatory NFκB inflammatory signalling pathway and NFκB dependant genes.

There are further, important differences between EDCs that are antiandrogenic or estrogenic. In contrast to estrogen induced inflammation, we demonstrate the long-term effects of transient *in utero* exposure to Vinclozolin failed to induce pre-malignancy. Inflammation and focal atrophy associated with increased proliferation has been described in human prostate specimens as proliferative inflammatory atrophy (PIA), and may be pre-malignant. The pathology arising from *in utero* Vinclozolin treatment and described herein does show inflammation and focal epithelial attenuation but in the absence of increased proliferation. Therefore it was concluded that Vinclozolin did not induce the pre-malignant lesion PIA.

Overall, the robust incidence of inflammation in 100% of young adult rats mimics more closely human non-bacterial prostatitis that occurs in young men. Ninety percent of prostatitis cases are of unknown cause and these data are the first to implicate anti-androgenic EDCs as a causative factor in the aetiology of this inflammatory disease of the prostate via activation of the classical NFκB inflammatory pathway. Whilst the level of Vinclozolin utilised in this study far exceeds that observed in the environment and projected human exposure, this study raises further concerns that *in utero* exposures to EDCs, with anti-androgenic activity have long range effects that include the development of prostatitis in early adult life, and provide further impetus to test the efficacy of treatments that block or abrogate NFκB signalling in the treatment of prostatitis.

Table 1: Effects of 6 day *in utero* administration of Vinclozolin at PND 0

Endpoint	Control	Vinclozolin
Dams assigned	16	16
Dams pregnant	12	13
Dams delivered late	0	0
Dam weight gain through dosing period (g)	49.29 ± 10.38	53.33 ± 11.81
Live litter sizes	12.18 ± 2.26	12.83 ± 2.33
M/F ratio at birth	1.10 ± 0.67	1.61 ± 1.80
Pup wt at birth (g)	6.44 ± 0.07	6.16 ± 0.09*
AGD in male offspring at birth	4.36 ± 0.08	3.75 ± 0.10*

Note: Litter mean ± SEM. AGD, anogenital distance; wt, weight; g, grams; M/F, Male to Female.

*p<0.05

Table 2: Effects of 6 day *in utero* administration of Vinclozolin on PND28 and PND56 reproductive organ weights

Endpoint	Control		Vinclozolin	
	PND 28	PND 56	PND 28	PND 56
Body (g)	70.97 ± 0.61	259.09 ± 7.21	73.6 ± 1.25	275.29 ± 5.02
AGD	25.55 ± 0.54	40.51 ± 0.91	24.20 ± 0.33*	30.82 ± 1.31*
VP (mg)	37.67 ± 1.45	223.74 ± 6.81	35.38 ± 1.25	199.16 ± 6.09*
AP (mg)	4.56 ± 0.55	69.32 ± 3.67	4.27 ± 0.32	82.23 ± 3.23*
LP (mg)	7.3 ± 0.49	45.68 ± 4.04	5.73 ± 0.61	56.69 ± 3.63
DP (mg)	9.1 ± 1.23	61.26 ± 4.42	9.15 ± 0.71	73.25 ± 3.98
SV (mg)	11.61 ± 0.96	507.94 ± 15.14	12.96 ± 0.58	373.15 ± 13.56*
Testis (g)	0.525 ± 0.02	2.41 ± 0.06	0.484 ± 0.01	2.58 ± 0.05

Note: Litter mean ± SEM. AGD, anogenital distance; VP, ventral prostate; AP, anterior prostate; LP, lateral prostate; DP, dorsal prostate; SV, seminal vesicle. *p<0.05

Table 3: Effects of 6 day *in utero* administration of Vinclozolin on key NFκB dependant inflammatory genes at post-natal day 56 compared to Control treatment

UniGene	Symbol	Description	Fold Difference
Rn.12300	Il1a	Interleukin 1 alpha	5.05
Rn.9869	Il1b	Interleukin 1 beta	2.02
Rn.1716	Il6ra	Interleukin 6 receptor, alpha	5.16
Rn.12138	Il6st	Interleukin 6 signal transducer	11.13
Rn.138115	Il8ra	Interleukin 8 receptor, alpha	7.80
Rn.90347	Il8rb	Interleukin 8 receptor, beta	4.29
Rn.92374	Il9	Interleukin 9	3.52
Rn.10045	Il9r	Interleukin 9 receptor	1.96
Rn.54465	Itgam	Integrin alpha M	3.34
N/A	LOC301289	Similar to Interleukin-17 precursor (IL-17) (Cytotoxic T lymphocyte-associated antigen 8) (CTLA-8)	13.43
Rn.2661	Mif	Macrophage migration inhibitory factor	9.19
Rn.10400	Nos2	Nitric oxide synthase 2, inducible	19.35
Rn.29157	Rac1	Ras-related C3 botulinum toxin substrate 1	9.89
Rn.40136	Tgfb1	Transforming growth factor, beta 1	27.30
Rn.107212	Tlr1	Similar to toll-like receptor 1 (LOC305354), mRNA	18.47
Rn.46387	Tlr2	Toll-like receptor 2	27.23

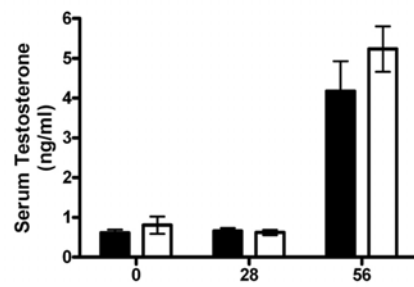
Rn.15273	Tlr3	Toll-like receptor 3	31.22
Rn.14534	Tlr4	Toll-like receptor 4	14.04
Rn.198962	Tlr5	Toll-like receptor 5	14.86
Rn.163249	Tlr6	Toll-like receptor 6	14.42
Rn.92495	Tlr9	Toll-like receptor 9	22.90
Rn.2275	Tnf	Tumor necrosis factor (TNF superfamily, member 2)	27.49
Rn.11119	Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	23.49
Rn.83633	Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b	29.62
Rn.30043	Tnfsf4	Tumor necrosis factor (ligand) superfamily, member 4	16.82
Rn.44218	Cd40lg	CD40 ligand	9.51
Rn.9868	Il10	Interleukin 10	0.44
Rn.50003	Il17b	Interleukin 17B	0.79
Rn.11118	Il18	Interleukin 18	0.92

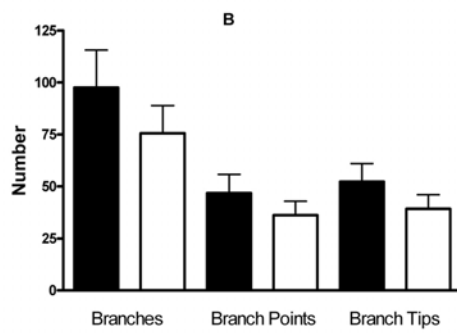
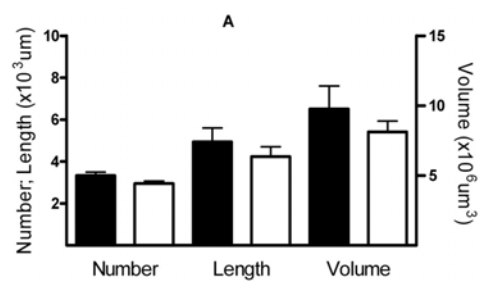
Figure 1: Absence of perturbations in branching morphogenesis in neo-natal off-spring exposed *in utero* to Vinclozolin. Analysis of (a) ductal number, length, volume or (b) branches, points or tips revealed no significant differences between Vinclozolin (open bar) and Control (solid bar) *in utero* exposed prostates. Mean $\pm$ SEM.

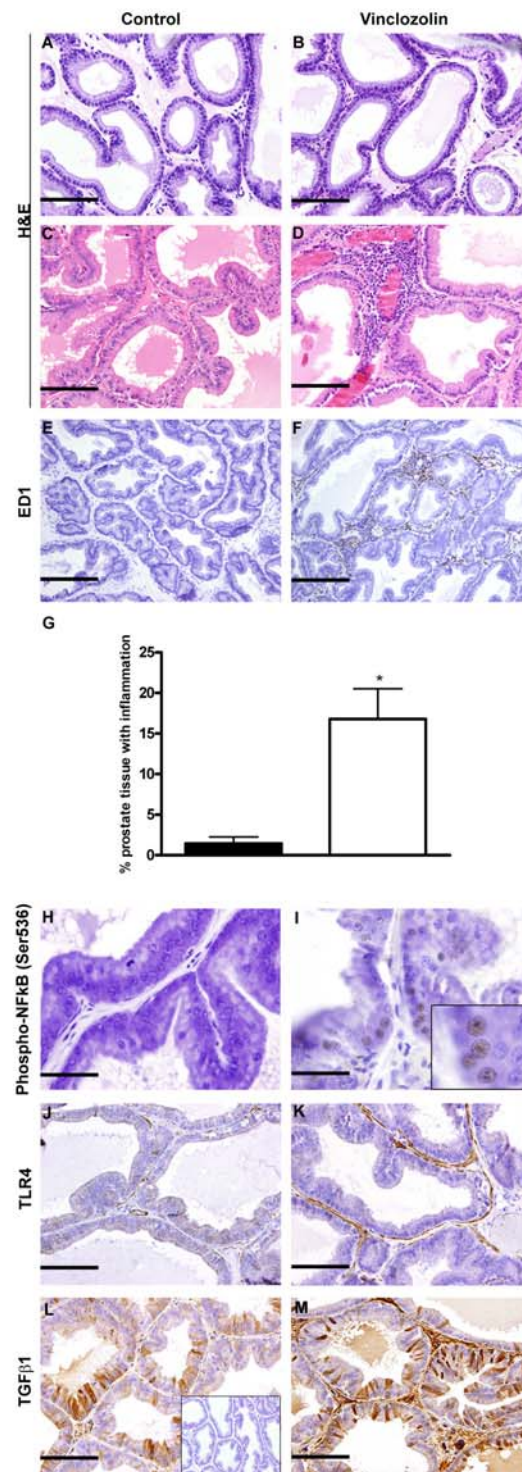
Figure 2: Serum Testosterone. No differences in serum testosterone between Vinclozolin (open bar) or Control (solid bar) *in utero* exposed at PND 0, 28 or 56. Mean $\pm$ SEM.

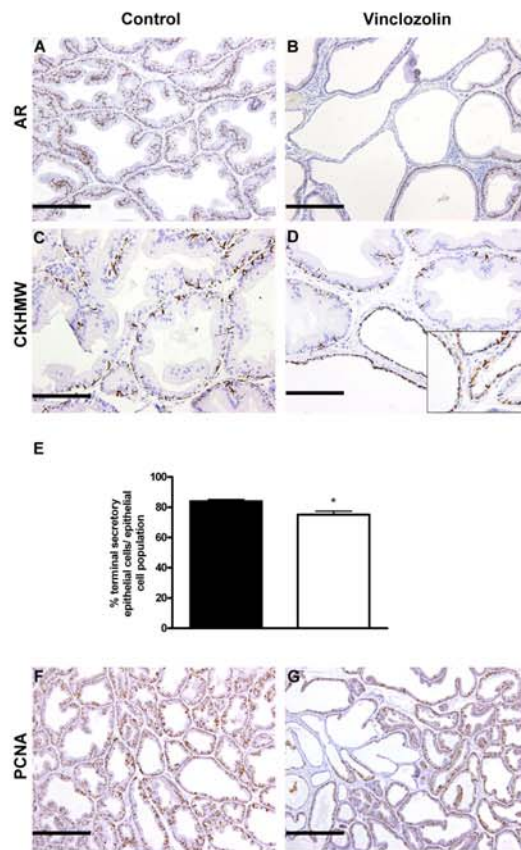
Figure 3: Early onset post-pubertal prostatitis in animals exposed *in utero* to Vinclozolin. Pre-pubertally no morphological differences between prostates of animals exposed *in utero* to Control (A) and Vinclozolin (B) were identified. Prostates from *in utero* exposed Control animals (C) showed extensive ductal branching and canalization with pseudo stratified columnar epithelial cells lining the ducts and continuous stromal sheaths surrounding the ducts. In *in utero* Vinclozolin exposed prostates (D) prominent, but focal, regions of inflammation are evident. Compared to Control *in utero* treatment (E) an increase in the proportion of macrophages surrounding the ducts and infiltrating into vessels is observed following *in utero* Vinclozolin treatment (F). Compared to control treatment (solid bar) a significant ($p < 0.05$) increase in the percentage of prostatic inflammation is observed following *in utero* Vinclozolin exposure (open bar) (G). Compared to *in utero* Control exposed tissues (H) activation of the inflammatory NF κ B pathway was evident in prostates following *in utero* Vinclozolin treatment with increased nuclear immuno-protein localisation of phospho-NF κ B p65 (Ser536) (H and inset). Compared to *in utero* Control exposed prostates (I), *in utero* Vinclozolin exposed prostates showed NF κ B dependant *TLR-4* gene expression up-regulation (J) and an increase in TGF β 1 expression (E, F). Bar 50 μ m (A-D, G, H, K, L); 20 μ m (E,F); 100 μ m (I, J). Mean $\pm$ SEM.

Figure 4: Epithelial attenuation in post-pubertal animals following *in utero* Vinclozolin treatment. In tissues exposed *in utero* to Control (A) AR is localised predominately to epithelial cells. (B) A down regulation in epithelial AR expression is observed in *in utero* Vinclozolin exposed prostates. (C) A discontinuous layer of basal cells were identified in tissues Control exposed by immuno-localisation of CKHMW, compared with a continuous layer in prostates exposed *in utero* to Vinclozolin (D). A significant loss of terminally differentiated epithelial was confirmed by stereological analysis (E; Solid bar, Control exposed *in utero*; Open bar, Vinclozolin exposed *in utero*). Proliferative activity was examined by immuno-localisation of PCNA (F, G). In attenuated glands of prostate tissues from animals exposed *in utero* to Vinclozolin a reduction in immuno-positive epithelial cells was observed (G). Bar 100 μm (A,B); 50 μm (C,D); 200 μm (F,G); Mean $\pm$ SEM * $p < 0.05$.









Appendix 2: Abstracts of Presentations

1. Southern Health Research Week, Clayton, Victoria Australia (Poster presentation)

Prostate disease begins in the womb: Early origins of prostatic inflammation caused by transient exposure to the endocrine disrupting chemical Vinclozolin.

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² *National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA, 27709*

The role of endocrine disrupting chemicals (EDCs) in the early origins of adult prostate disease is of concern and controversy to the lay and scientific communities. Environmental estrogens disrupt development and have been linked to the increased incidence of prostate disease in aging men. This study is the first to implicate the EDC Vinclozolin (an anti-androgen) as a causative factor. Early-onset prostate inflammation is evident in 100% of post pubertal (56 days) male off-spring exposed orally *in utero* during the sensitive period of male reproductive tract development (gestational days 14-19). Prostatic inflammation is due to activation of the NFκB inflammatory pathway with phosphorylation and activation of NFκB evident by increased nuclear translocation of phospho-NFκB p65 (Ser536). Pro-inflammatory NFκB dependant genes are also activated in post-pubertal animals following *in utero* Vinclozolin treatment including the chemokine Interleukin-8, which is associated with the increased

macrophage infiltration, and the cytokine Transforming Growth Factor (TGF)- β 1, associated with the down regulation of the anti-inflammatory gene Interleukin-10. These are the first data to implicate EDC exposure as a causative factor of prostatic inflammation and, given the link between chronic inflammation and prostate cancer, warrant further regulatory investigation.

Prostate disease begins in the womb: Early origins of prostatic inflammation caused by transient exposure to the endocrine disrupting chemical (EDCs) – Vinclozolin.

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EDC exposure as a causative factor of prostatic inflammation and, given the link between chronic inflammation and prostate cancer, warrant further regulatory investigation.

3. Healthy Start for a Healthy Life: The Wintour's Tale, A Satellite Conference of DOHaD. Melbourne, Australia (Oral Presentation)

Prostate Inflammatory Atrophy induced following transient *in utero* exposure to the endocrine disruptor Vinclozolin

Prue A. Cowin¹, Paul Foster², Stephen J. McPherson¹ and Gail P. Risbridger¹

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Fetal exposure to the anti-androgenic fungicide Vinclozolin has been shown to have adverse effects on male reproductive tract development. Recent studies have suggested the adult prostate may also be altered by exposure to Vinclozolin. However, it is not clear whether the reported prostatic pathology occurs earlier in development, nor is it clear whether growth regulatory pathways may be affected. Therefore, the aim of this study was to determine the effects of transient *in utero* exposure to Vinclozolin on the pre- and post-pubertal rodent prostate gland.

Fetal rats were exposed to Vinclozolin (100mg.kg bw) or corn oil vehicle control (2.5ml.kg bw) *in utero* for 6 days from gestational day 14 to 19 via oral administration to pregnant dams. This period corresponds to a critical window of male reproductive tract development, particularly the onset of appearance of androgen receptors in the urogenital sinus and the beginning of prostate budding. Male

pups were aged to 4 or 8 weeks (pre-pubertal; post-pubertal respectively) before tissue was collected for analysis. At 4 weeks of age *in utero* exposure to Vinclozolin resulted in no significant developmental or morphological abnormalities compared to control animals. Prostates of Vinclozolin-treated post-pubertal animals displayed apparent epithelial atrophy which was confirmed by subsequent stereological analysis and immunohistochemistry revealed a significant increase in the percentage of basal cells within epithelia of atrophic glands. Analysis of hormone receptor expression revealed reduced epithelial and increased stromal AR expression in Vinclozolin-treated tissues, although no differences in estrogen receptor alpha or beta expression were observed. An apparent increase in inflammatory cells was observed in Vinclozolin treated tissues and preliminary studies suggest a link with up regulation in the NFκB signaling pathway.

Overall, this study demonstrates that transient *in utero* exposure to an anti-androgenic chemical during a critical period of male reproductive tract development has the potential to induce prostate inflammatory atrophy by disrupting normal prostate development and inducing an inflammatory response which only becomes identifiable in post-pubertal animals implying aberrant androgenic response. This work is of particular significance as there is increasing literature suggesting a link between chronic inflammation and prostate cancer.

4. 50th Annual Scientific Meeting, Endocrine Society of Australia, Christchurch, New Zealand. (Oral Presentation)

Prostate Inflammatory Atrophy induced following transient *in utero* exposure to the endocrine disruptor Vinclozolin

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5. 50th Annual Scientific Meeting, Endocrine Society of Australia, Christchurch, New Zealand.
(Seminar Presentation)

Transient endocrine disruption induces prostate pathologies upon aging

Risbridger GP¹, Cowin PA¹, McPherson SJ¹, Foster P²

¹*Centre for Urological Research, Monash Institute of Medical Research, Monash University, Melbourne, VIC, Australia*

²*Research Triangle Park, National Institute of Environmental Health Sciences, North Carolina, United States*

Normal development and differentiation of the prostate gland is regulated by androgenic and estrogenic hormones requiring a complex interplay between endocrine and cell-cell signaling. Although prostate disease occurs in later life, it is known that transient perturbations in hormone action or in the relative ratio of androgens to estrogens, result in pathologies in late life – long after the initial event. We and others have demonstrated the importance of androgens and estrogens in the maintenance of the epithelial stem cell niche and the pivotal role of the stroma in mediating these effects.

The fungicide Vinclozolin is an anti-androgen and an endocrine disrupting chemical (EDC), with adverse effects on male reproductive tract development. We show transient neonatal exposure to Vinclozolin results in the perturbation of hormone action and aberrant stromal-epithelial cell signaling that alters the prostatic stem cell niche. Epithelial cell pathologies occur at maturity; specifically prostatic inflammatory atrophy. Since chronic inflammation is linked to the onset of premalignant lesions, these results provide a mechanism for the long range effects of transient exposure to Vinclozolin on the prostate gland.

Appendix 3: Updated Biographical Sketch for Prof. Gail P. Risbridger

BIOGRAPHICAL SKETCH

NAME Gail Risbridger		POSITION TITLE Professor	
EDUCATION/TRAINING			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
University of Sussex (UK)	B.Sc (Hons)	1971-1974	Biochemistry
Oxford University	Dip. Ed.	1974-1975	Education
Universtiy of Strathclyde (Glasgow)	M.Sc.	1976-1977	Reproductive Endocrinology
Monash University (Australia)	PhD	1977-1980	Reproductive Endocrinology

RESEARCH AND PROFESSIONAL EXPERIENCE:

Previous Employment:

1980-1981	Research Officer, NH&MRC (National Health and Medical Research Council), Royal Children's Hospital, Melbourne.
1981-1990	Senior Research Officer, Research Fellow (NH&MRC), Departments of Physiology & Anatomy, Monash University
1988-1994	Senior Lecturer, Dept Anatomy
1991-1995	NH&MRC Senior Research Fellow, Institute of Reproduction & Development, Monash University
1996	NH&MRC Principal Research Fellow, Institute of Reproduction & Development, Monash University
1998 -2001	Associate Professor, Faculty of Medicine, Monash University

Current Positions:

1996	NH&MRC Principal Research Fellow, Monash Institute of Medical Research, Monash University
1997	Associate Director Monash Institute of Medical Research
1996	Director for Centre for Urological Research, Monash Institute of Medical Research, Monash University
2000	Executive Committee Member, Australian Federal Government Centre for Excellence in Male Reproductive Health, Andrology Australia.
2001	Professor, Faculty of Medicine, Nursing and Health Sciences, Monash University
2006	Associate Dean, Research Centre & Institutes, Faculty of Medicine, Nursing and Health Sciences, Monash University

Other Experience and Professional Memberships

Federal Government public advisory committees.

- 1991-1997 NH&MRC, Regional Grants Interviewing Committee
- 1995-1997 NH&MRC Assigner's Panel Member
- 1997 - NH&MRC Program Grant Interviewing Committee
- 1998-2000 NH&MRC Chair of Discipline panel for Endocrinology & Reproduction,
- 2005- NH&MRC Member of Grant Review Panels: Endocrinology, Cancer Biology
- 2006- NH&MRC Member of Career Development Awards Panel
- 2001 - Andrology Australia, Member of Executive Committee of Management
- 2005 - Prostate Cancer Foundation of Australia. Member of Executive Scientific Advisory Group
- 2005 - Australian Prostate Cancer Consortium - APCC, Executive Committee of Management
- 2006- Medical & Scientific Committee of the Cancer Council Victoria, Member

Editorial Boards

- 1992-1995 Member of Editorial Board, Molecular & Cellular Endocrinology
- 2001-6 Member of Editorial Board, Endocrinology
- 2001- Section Editor, Reproductive Biology; Cell and Tissue Research
- 2003- Section Editor, Journal of Molecular Endocrinology

International & National Endocrine Society Positions

- 1994 -1998 Endocrine Society of Australia, Council Member and Secretary
- 2005 - Women in Endocrinology Board member and Awards Committee member
- 2006-8 Member of Steering Committee for US Endocrine Society Meetings

University Positions

- 1993 - Member of Directors Management Group, Monash Institute of Medical Research
- 1994-1996 Member of Bachelor of Medical Science Committee, Faculty of Medicine, Monash University.
- 1995 -1997 Member of Senior Women's Advancement Scheme, Vice Chancellors/Equal Opportunity office
- 1996 -1998 Member Strategic Planning Committee, Monash Institute of Medical Research
- 1996 -1998 Chairman, OHS Committee Zone 11, Monash University
- 1996 -1999 Member, Faculty Medicine Affirmative Action Committee
- 1993 - 2000 Member of Standing Committee on Animal Services, Monash University
- 1997 - Member of Advisory Board Subcommittee for Commercialization
- 1998-2000 Member, Faculty of Medicine Research Committee
- 2000- 6 Chair of the MIRD Postgraduate Student Committee
- 2000-2003 Co-ordinator BSc Honours Program MMC4000
- 2001-2003 Member Faculty Research Degrees Committee
- 2004 - Member Faculty of Medicine Research Management Committee
- 2006- Deputy Chair, Faculty Research Committee
- 2006-8 Associate Dean, Research Centres and Institutes

Honours & Awards

- 2006 Asia-Oceania Medal - British Endocrine Society (for Contribution to Endocrinology)
- 2006 Postgraduate Supervision Award, Monash University, Special Commendation,

2005 Industry Engagement Award, Monash University
 2003 Fulbright Senior Scholar Award - Department of Urology, Columbia University, New York
 2001 Monash - Kings College Fellowship - London
 2000 Silver Jubilee Prize – Monash University, Faculty of Medicine
 1997 Academy of Science - Royal Society Exchange Program Award.
 1991 British Council: Academic Links and Interchange Scheme Award
 1991 Finnish Academy Science Award Visiting Scientist
 1977-1979 Monash University Graduate Scholarship
 1975-1977 MRC (UK) Postgraduate Scholarship

Successful Grants

Major National Grants

2006- NH&MRC Fellowship Grant & SEO, ID 384104 - **Risbridger**
 Role: PI Funding: \$130,000 p.a.
 2005 – 2007 NH&MRC Program Grant Control of Reproductive Processes ID 334011 - De Kretser, **Risbridger**, Hedger, Hearn, Jenkin, Loveland, O'Bryan, Wallace
 Role: Co-PI Funding: \$1,545,075 p.a.
 2005 – 2009 NH&MRC Enabling grant Australian Prostate Cancer Collaboration (APCC) Bio-Resource (currently known as the Commonwealth Bank Australian Prostate Cancer BioResource in partnership with Andrology Australia) ID 290542 - Clements, Tilley, Sutherland, **Risbridger**
 Role: CI Funding: \$2,100,000

Major International Grants

2008-2010 **Risbridger**, Taylor
 USA Army Medical Research & Material Command (DOD) Grant
 Idea Development Award #PC073444 Using Human Stem Cells to Study the Role of the Stroma in the Initiation of Prostate Cancer
 Role: PI
 Funding: US\$154,882.37/year
 2008 – 2010 Stuart Ellem, **Risbridger**
 USA Army Medical Research & Material Command (DOD) Grant
 Prostate Cancer Training Award #PC073307 Linking Estrogen, Prostatitis and Prostate Cancer
 Role: Mentor
 Funding: Total US\$144,480
 2007 **Risbridger**
USA Army Medical Research & Material Command (DOD) Grant Concept Award #PC060377 Endocrine Disruption and Human Prostate Cancer
 Reports on a novel, reliable, and reproducible model system that can be used to study human prostate development and maturation and that can be used to test the potential effects of endocrine disruption chemicals (EDCs) on human prostate tissues.
 Funding: Total \$US96,644
 2006- present **Risbridger**, de Kretser, Simpson & McLachlan, O'Bryan
Schering AG
Melbourne Male Network is a joint venture between MIRD Centre for Urological Research and Prince Henry's Institute of Medical Research & Division of Andrology &

Gynecology, Schering AG. It was funded following a bid for funds from the International Pool of Funds for Corporate Research at Schering AG.

Role: Lead PI Funding: AUD\$1,061,855

2007-2008 **Balanathan & Risbridger**
USA Army Medical Research & Material Command (DOD) Grant ID New Investigator Award #PC060112

New Action of Inhibin Alpha Subunit in Advanced Prostate Cancer.

To investigate a potentially new role for inhibin alpha subunit (INHA) in promoting prostate cancer growth and metastasis.

Funding: Total \$US113,614

2006-2008 **McPherson & Risbridger**
USA Army Medical Research & Material Command (DOD) Grant ID New Investigator Award #PC050653

Is hormonal induction of prostate carcinogenesis due to declining androgens in late life and/or increased estrogen in early life?

Role: CoI

Total Funding: \$US219,334

Other Grants:

2008 - 2010. **Prostate Cancer Foundation Australia (PCFA) & Cancer Australia (Co-Funded) NHMRC/PCFA Priority Call Grant “MicroRNAs in prostate cancer; Novel biomarkers and potential therapeutic targets”**

Role: CoI Funding: \$648,000

2008 - 2011 **Prostate Cancer Foundation Australia (PCFA) Young Investigator Grant.** Project ID: PCFAY01 “Molecular profiling and plasticity of prostate cancer stem cells with disease progression”

Role: Mentor Funding: \$300,000

2008 – 2009 **GlaxoSmithKline Australia – GSKA Post Graduate Support Grant.** New action of inhibin alpha subunit in advanced prostate cancer

Role: Mentor Funding: \$25,000

2008 **Faculty of Medicine, Nursing & Health Sciences, Monash University – Monash Strategic Grants.** Project ID: ECD040 “ Biological Mechanisms underlining the tumour suppressive and pro-metastatic role of inhibin- α subunit in the changing tumour microenvironment

Role: Mentor Funding: A\$35,000

2007 **Australian Research Council (ARC) - Linkage Infrastructure, Equipment and Facilities Scheme.** Project ID: LE0883078 “Liquid Chromatography Tandem Mass Spectrometry Steroid Analysis Facility.”

Role: CoI Funding: \$356,000

2007 **ANZ Trustees – Medical Research & Technology in Victoria – The William Buckland Foundation.** Dual – or multi-functionality of inhibin- α subunit in prostate cancer progression

Role: Mentor Funding: \$15,000

2007 **The Cancer Council Victoria – Cancer Research Vacation Studentship.** Endocrine Disrupting chemicals and early origins of prostate cancer

Role: Mentor Funding: \$1,500

2007 **Monash University – 2007 Near Miss Grant for NHMRC Projects – Deputy Vice Chancellor (Research)**

Role: PI Funding: \$10,000

- 2007 **Monash University – 2007 Near Miss Grant for NHMRC Projects – Faculty of Medicine, Nursing & Health Sciences**
Role: PI Funding: \$10,000
- 2007 – 2010 **The Coulson Group Pty Ltd – “PhD Scholarship”. Risbridger, G**
Role: PI Funding: \$81,000
- 2007 – **Harold and Core Brennen Benevolent Trust – The Role of Cancer Associated Fibroblasts (CAFs) in Prostate Carcinogenesis**
Role: CI Funding: \$30,000
- 2007 – **ANZ Trustees – James & Vera Lawson Trust – The role of cancer associated fibroblasts (CAFS) in prostate carcinogenesis**
Role: PI Funding: \$10,000
- 2006-2008 **Cancer Council Victoria Project Grant: Early origins of prostate cancer. Risbridger G, McPherson S**
Role: PI Funding: \$203,000
- 2006 **Dorothy Hill Memorial Trust “Prof. G Cunha International Visiting Fellowship”**
Role: PI Funding: \$12,800
- 2006 – **Fuji-Xerox Community Services Award – Equipment Grant : Double Header Microscope Attachment**
Role: PI Funding: \$5,000
- 2006 - **The Eirene Lucas Foundation - Equipment Grant : Double Header Microscope Attachment**
Role: PI Funding: \$9,000
- 2006 - **H&L Hecht Trust – Role of Stroma in Carcinogenesis**
Role: PI Funding: \$20,000
- 2006 **Thomas & Rosalinda Ditchfield Medical Research Trust: “Is activin C a marker of early stage liver disease?” Risbridger G, Gold E.**
Role: PI Funding: \$12,800
- 2006 **J & R McGauran Trust.”Is activin C a marker of early stage liver disease?” Risbridger G, Gold E.**
Role: PI Funding: \$14,500
- 2006 **Oliver-Affleck Fund “Is activin C a marker of early stage liver disease?” Risbridger G, Gold E.**
Role: PI Funding: \$17,200
- 2005 – 2006 **Helen McPherson Smith Trust – Investigation of the Biological effects of increased activin C. Risbridger, G and Gold, E.**
Role: PI Funding: \$35,000

Publications

From a total of >130 publications, the following lists those since 2000

1. Taylor RA, **Risbridger GP** (2008). The path towards identifying prostatic stem cells. Differentiation. (In Press) **(IF 3.7)**
2. McPherson SJ, Ellem SJ, **Risbridger GP** (2008). Estrogen regulated development and differentiation of the prostate. Differentiation. (in press) **(IF 3.7)**
3. Hung T-T, Wang H, Kingsley E, Risbridger GP, Russell PJ (2008). Molecular profiling of bladder cancer: Involvement of the TGF- β pathway in bladder cancer progression. Cancer Letters **(IF 3.27)** (In press)
4. Taylor RA, **Risbridger GP** (2008). Role of the tumour stroma in prostate cancer. Current Cancer Drug Target (In Press) **(IF 5.7)**

5. Cowin, P, Foster P, Pedersen J, Hedwards S, McPherson S, **Risbridger G** (2008). Early onset endocrine disruptor induced prostatitis in the rat. *Environmental Health Perspectives* (In Press) **(IF5,861)**
6. Ilic D, Egberts K, McKenzie J, **Risbridger G**, Green S. 2008. Informing men about prostate cancer screening; a randomized controlled trial of patient education materials. *Journal of General Internal Medicine* **(IF 2.9)** (In press)
7. Ellem SJ & **Risbridger GP** (2007) Treating Prostate Cancer; A rationale for targeting local estrogens. *Nature Review cancer*. (7): 621-627 **(IF 31.6)**
8. **Risbridger GP**, Ellem SJ, McPherson SJ (2007). Estrogen action on the prostate gland: A critical mix of endocrine and paracrine signalling. *Journal of Molecular Endocrinology*. *J Mol Endo*, 39(3)L 183-8 **(IF 3.0)**
9. **Risbridger, GP**, Butler C (2007). Activins and inhibin in cancer progression in Transforming Growth Factor-Beta in Cancer Therapy, Volume 1: Basic and Clinical Biology. Ed: Sonia B. Jakowlew, Humana Press. (26):411-424
10. Cowin PA, Foster P, **Risbridger, GP** (2007). Endocrine Disruption in the Male, *Endocrine disrupting Chemicals: From Basic Research to Clinical Practice*, (3): 33-62, Human Press
11. Ellem SJ & McPherson SJ, Patchev V, Fritzmeier KH and **Risbridger, GP**, (2007). The Role of ER Alpha and Beta In the Prostate. Insights From Genetic Models and Isoform-Selective Ligands, *Tissue-Specific Estrogen Action: Novel Mechanisms, Novel Ligands, Novel Therapies?* Ernst Schering Foundation, Pringer-Verlag, p. 131-147
12. McPherson, SJ, Ellem SJ, Simpson, ER, Patchev, V, Fritzmeier, K-H, **Risbridger, GP** (2007) Essential role for estrogen receptor β in stromal-epithelial regulation of prostatic hyperplasia. *Endocrinology*. 148(2):566-74 **(IF 5.3)**
13. Ricke WA, McPherson SJ, Bianco JJ, Cunha GR, Wang Y, **Risbridger GP**. 2007. Prostatic hormonal carcinogenesis is mediated by in situ estrogen production and estrogen receptor alpha-signaling. *FASEB Journal* **(IF 6.7)** (E-Publication)
14. **Risbridger, GP**, Frydenberg, M. 2006 *Endocrinology of Prostate Cancer*, In *Endocrinology*. Editor: Leslie J. De Groot. 5th edition Elsevier, W.B. Sanders, Philadelphia, PA. p3325-3337
15. **Risbridger, GP**, Taylor R. 2006 The physiology of the male sex accessory tissues. In *Physiology of Reproduction*, Editor J. D. Neill. 3rd edition. Elsevier, San Diego, CA. P1149-1172
16. **Risbridger, Gail P**. Activins. 2006 *Encyclopaedia of Hormones. Growth Factors and Cytokines*. Editors: Elsevier Press, Academic Press, San Diego
17. **Risbridger, GP**. and Butler, C, 2006 Activins and Leydig cell development, differentiation and disease (Chapter 22) from *Contemporary Endocrinology*. In *The Leydig Cell in Health and Disease*, p323-331, Humana Press.
18. Ellem SJ & **Risbridger, GP** (2006) Aromatase and prostate cancer. *Minerva Endocrinologica* 31 (1) 1-12
19. Taylor RA, Cowin, Couse JF, Korach KS, **Risbridger GP** (2006) 17 β -estradiol induces apoptosis in the developing rodent prostate independently of ER α or ER β . *Endocrinology* 147(1): 191-200 **(IF5.2)**
20. Taylor RA, Cowin, PA., Cunha GR, Pera M, Trounson AO, Pedersen J, **Risbridger GP**. (2006) Formation of human prostate tissue from embryonic stem cells. *Nature Methods* 3 (3):179-181 **(IF15)**
21. Bianco JJ, McPherson SJ, Wang H, Prins GS, **Risbridger GP**, (2006) Transient neonatal estrogen exposure to estrogen deficient mice (Aromatase knockout) reduces prostate weight and induces inflammation in late life. *Am J Pathol*. 68(6):1869-78 **(IF 5.9)**.

22. **Risbridger GP**, Drummond A, Wlodek M. (2005) Editors of Special Issue Cell & Tissue Research on Development & Disease in Reproduction
23. Butler CM, Gold EJ, **Risbridger GP**. (2005) Should activin bC be more than a fading snapshot in the activin/TGFb family album? Cytokine & Growth Factor Reviews 16 (4-5): 377-85 **(IF 11.5)**
24. Almahbobi G, Hedwards, S, Fricout G, Jeulin D, Bertram JF, **Risbridger GP**. 2005 Computer-based detection of early changes to branching morphogenesis reveals multiple mechanisms of prostate enlargement. Journal of Pathology 206:52-61 **(IF 5.8)**
25. Gold EJ, Zhang X, Wheatley AM, Mellor SL, Cranfield M, **Risbridger GP**, Groome NP, Fleming JS. 2005 β A- and β C-activin, follistatin, activin receptor and mRNA and β C-activin peptide expression during rat liver regeneration. Journal of Molecular Endocrinology Apr; 34(2):505-15. **(IF 4.3)**
26. **Risbridger GP**, Almahbobi GA, Taylor RA (2005) Early prostate development and its associate with late life prostate disease. Cell and Tissue Research Oct;322(1):173-81**(IF2.6)**
27. Ilic D, **Risbridger G**, Green S. 2005 The informed male: what do men want to know about prostate cancer screening? International Journal on Mens Health and Gender 2,4:414-420.**(IF not available)**
28. Simpson ER, McPherson S, Jones M, Robertson K, Boon WC, **Risbridger, G**. 2004 Role of estrogens in the male reproductive tract In: New molecular mechanisms of estrogen action and their impact on future perspectives in estrogen therapy. Ernst Schering Research Foundation Workshop 46. Edited by KS Korach, A. Hillisch, FH Fritzemeier. Springer-Verlag Berlin. p89-125
29. Cunha GR, Rieke W, Thomson A, Marker PC, **Risbridger GP**, Hayward S, Wang YZ, Donjacour AA, Kurita T(2004) Hormonal, cellular and molecular regulation of normal and neoplastic prostatic development. Journal of Steroid Biochemistry & Molecular Biology Nov;92(4):221-36. **(IF 2.8)**
30. Ball, E.M.A., Mellor, S. L., **Risbridger, G.P**. 2004 Cancer progression: is inhibin α from Venus or Mars? Cytokine and Growth Factor Reviews. 15, 5, 291-296. **(IF 11.5)**
31. **Risbridger GP**, Shibata A, Ferguson KL, Stamey TA, McNeal JE, Peehl DM. 2004. Elevated expression of inhibin α in prostate cancer. Journal of Urology. 171, 192-196 **(IF 3.9)Cited 4**
32. Balanathan P, Ball EMA, Wang H, Harris SE, Shelling AN, **Risbridger GP**, 2004 Epigenetic regulation of Inhibin alpha subunit gene in prostate cancer cell lines. Journal of Molecular Endocrinology. 32 (1): 55-67**(IF 4.3)Cited 3**
33. Ellem S, Schmitt J, Pedersen J, Frydenberg M, **Risbridger G**. 2004 Local aromatase expression in human prostate is altered in malignancy. Journal of Clinical Endocrinology and Metabolism;89(5):2434-41 **(IF 5.8)Cited 7**
34. **Risbridger GP**, Jarred RA, Wang H, Trounson AO 2004 All power to the prostatic stroma. ANZ Journal of Surgery, Volume 74(3): A1-A8
35. Gold E.J., O'Bryan M.K., Mellor S.L., Cranfield M., **Risbridger G.**, Groome P., and Flemming J.S., 2004. Cell-specific expression of β C-activin the rat reproductive tract, adrenal and liver. Mol Cell Endocrinol. 222(1-2):61-9. **(IF 2.9)**
36. Ilic D, **Risbridger G**, Green S. 2004 Searching the internet for information on prostate cancer screening: an assessment of quality. Urology (64) 1, p112-116 **(IF 2.7)**
37. **Risbridger G**, Ball E, Wang H, Mellor S, Peehl D. 2004 Re-evaluation of inhibin α subunit as a tumour suppressor in prostate cancer. Mol Cell Endocrinol, 225, 73–76 **(IF 2.9)**
38. Ball EMA, **Risbridger, GP** 2003 Stem cells in kidney morphogenesis *in* Stem Cells Handbook, ed Stuart Sell, Humana Press.
39. Ball EMA, **Risbridger GP**, 2003 New perspectives on Growth Factor-sex steroid interaction in the prostate Cytokine & Growth Factor Reviews, Vol 14/1 p 5-16 **(IF 11.5)Cited 1**
40. Mellor SL, Ball EMA, O'Connor AE, Ethier J-F, Cranfield M, Schmitt JF, Phillips DJ, Groome NP, **Risbridger GP**. 2003 Activin β_c subunit heterodimers provide a new mechanism for regulating activin levels in the prostate. Endocrinology, 144 (10) 4410-4419 **(IF 5.2)Cited 3**

41. Jarred RA, McPherson SJ, Jones MEE, Simpson ER, **Risbridger GP** 2003. Anti-androgenic action by red clover-derived dietary isoflavones reduces non-malignant prostate enlargement in aromatase knockout (ArKO) mice. *The Prostate*, 56 (1): 54-64 **(IF 3.7) Cited 2**
42. Gold EJ, Francis RJB, Zimmermann A, Mellor SL, Cranfield M, **Risbridger GP**, Groome NP, Wheatley AM, Fleming JS. 2003 Changes in activin and activin receptor subunit expression in rat liver during the development of CC14-induced cirrhosis. *Molecular and Cellular Endocrinology*, (201) 1-2, 143-53 **(IF 2.9) Cited 2**
43. **Risbridger GP**, Bianco JJ, Ellem SJ, McPherson SJ 2003. Oestrogens and prostate cancer. *Endocrine-Related Cancer*. 10; 187-191 **(IF 4.7)Cited 18**
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