MUT/2016/05

COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT (COM)

Environmentally induced epigenetic toxicity: potential public health concerns

A presentation by Dr Emma Marczylo, PHE

Questions:

Do Members have any questions or comments on the topics raised in Dr Marczylo's presentation?

Aspects for discussion

- **Standard Methods – depending on the purpose of the investigations (experimental and bioinformatics)**
- **Understanding what is 'normal' and human variation**
- **Better use of existing biosamples from regulatory studies**
- **Appropriate doses for human risk assessment**

Epigenetics: the transgenerational effects of Vinclozolin

The paper provides a brief overview of the role of epigenetics in mediating transgenerational effects and provides summaries of studies investigating effects induced by vinclozolin, which follows on from the initial COM review in 2006. It is hoped that this example will facilitate discussions on how epigenetic and transgenerational changes should be evaluated and the plausibility of developing risk assessment strategies. A number of questions are given for Members consideration.

What are Members opinions of the Vinclozolin studies provided?

- Comments on protocols, methodologies, end points
- Are the effects reported consistent in comparable studies? If not, what are the possible reasons for inconsistencies.
- How can the mechanistic and transcriptomic studies, such as those published by Skinner et al, be used for hazard characterisation?
- Do studies like these provide any new information which would warrant amending current risk assessment strategies?
-

What are Members opinions of the use of epigenetics in human risk assessment?

- Should demonstration of epigenetic or transgenerational effects impact on risk assessment?
- How can the relevance of epigenetic (eg methylation) changes *in vitro* and in animal models be assessed?

- Should a battery of studies/protocols be used to identify and characterise a potential epigenetic hazard?
- Inter-individual variation and species differences - greater or less than for other endpoints.
- Can relevant epigenetic effects be identified in other toxicity tests?

- How could you evaluate the effects of multiple exposures (eg to endocrine disruptors) on the epigenome?
- What are the relevance of truly transgenerational (ie to F3) effects to human risk assessment?

To which aspects of epigenetics risk assessment can COM contribute?

How could COM work with other Committees to provide guidance or establish testing strategies?

Secretariat/PHE Toxicology Unit

May 2016

COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT (COM)

ANNEX 1:

MUT/2016/05: Transgenerational effects of methylation

Brieño-Enríquez MA, García-López J, et al 2015 Exposure to endocrine disruptor induces transgenerational epigenetic deregulation of microRNAs in primordial germ cells. PLoS One. 2015 Apr 21;10(4):e0124296.

Guerrero-Bosagna C, Settles M, Lucker B, Skinner MK. (2010) Epigenetic transgenerational actions of vinclozolin on promoter regions of the sperm epigenome. PLoS One. 2010 Sep 30;5(9). pii: e13100.

Guerrero-Bosagna C, Covert TR, Haque MM, Settles M, Nilsson EE, Anway MD,Skinner MK. Epigenetic transgenerational inheritance of vinclozolin induced mouse adult onset disease and associated sperm epigenome biomarkers. Reprod Toxicol. 2012 Dec;34(4):694-707.

Inawaka K(1), Kawabe M, Takahashi S, Doi Y, Tomigahara Y, Tarui H, Abe J, Kawamura S, Shirai T. (2009) Maternal exposure to anti-androgenic compounds, vinclozolin, flutamide and procymidone, has no effects on spermatogenesis and DNA methylation in male rats of subsequent generations. Toxicol Appl Pharmacol. 2009 Jun 1;237(2):178-87.

Stouder C, Paoloni-Giacobino A. (2010) Transgenerational effects of the endocrine disruptor vinclozolin on the methylation pattern of imprinted genes in the mouse sperm. Reproduction. 139(2):373-9.

MUT/2016/05

Epigenetics: the transgenerational effects of Vinclozolin

Introduction

1) Transgenerational[1] epigenetic inheritance is defined as the transmittance of information from one generation of an organism to the next, via the germ cells, and has the potential to affect offspring without alteration of the primary structure of DNA. Epigenetic mechanisms which contribute to these alterations are broadly characterized as; changes to DNA methylation status, histone modifications and perturbation of micro(mi)RNA's. A preliminary evaluation of the role of methylation status and transgenerational effects of methylation was undertaken by the COM in October 2006 (MUT/06/15 – ANNEX 1). This was in response to the Medical and Toxicology panel of the Advisory Committee on Pesticides (ACP), which had requested consideration of this topic, in particular the recent publication on the potential for vinclozolin (VZ) and methoxychlor to induce transgenerational effects in rats via the male line, following exposure of pregnant females (Anway et al 2005). Decreased spermatogenic capacity and reduced fertility were reported over four generations after high (100 mg/kg/day) intraperitoneal (ip) doses during embryonic days (ED) 8 -14. After discussion of the paper, the COM suggested that VZ could be used as a model compound to further investigate gene changes in relation to toxicological outcome. More generally, it was commented that a microarray approach to analysis could be used to examine the effects of chemicals and methylation on specific gene expressions e.g. whether up regulated or down regulated. It was also concluded that DNA methylation effects would be a very important area for future research for a potentially wide range of toxicological effects, particularly for carcinogenesis.

2) A preliminary evaluation of the role of epigenetics in carcinogenesis was undertaken by COC in 2013 (CC/2013/05 & 06) – arsenic and benzene were examined. It was noted that at the experimental level there were significant data gaps, and that many of the studies investigating epigenetic endpoints had not been replicated. The human relevance of animal studies was not clear and often epidemiological evidence was not available. Overall the Committee considered that it was possible that epigenetic changes contribute to carcinogenicity for arsenic and benzene, but much more work would be required to more clearly define their role. It was also noted that it would not always be evident when the epigenetic changes were causal for tumour development or an effect of tumour development.

3) COM considered the topic of epigenetics and epimutations at Horizon scanning exercises (2013 and 2015) and Members expressed an interest in the topic with view to examining its importance in future risk assessment strategies. Whilst it was considered that epigenetic changes would likely be more important in carcinogenesis, it was noted that if epimutations occur in germ cells and are heritable, then this could potentially induce transgenerational effects which would generate a concern worth investigating. To bring Members up to date with explorations in this area, Dr Emma Marcelzyo has agreed to give a presentation on the recent and ongoing work at PHE and

[1] The term is often used to include any effects seen in any generation of offspring but true transgenerational effects are considered to be the first generation not exposed in utero (F3) –

this will provide a thorough background of current research and opinions in this area, in particular examination of current evidence of epigenetic changes in human studies (her publication is attached ANNEX 1).

4) This paper introduces some general theory, and examines the concept of transgenerational epigenetics, using VZ as the example, with view to understanding the potential impact of chemically induced changes and possible risk assessment. Many of the studies published since the original Anway appear (2005) have been conducted by the same group of researchers [Skinner et al], using the identical dosing and breeding regime. These studies have generally set out to provide further evidence for their original claims and to extend their understanding of the potential effects and mechanisms underpinning the transgenerational phenomenon. There are a few publications from other laboratories examining different endpoints or using mice. The publications considered are generally very data rich and it is not always straightforward to compare studies with one another or to develop a cohesive overview of the mechanism underpinning the effects. Some detail of the studies are presented in a tabulated summary format (Tables 1 and 2 – ANNEX 2)

Transgenerational epigenetics – in brief

5) Early research into transgenerational epigenetics recognised the importance of phenotypic alterations attributable to environment, chemical exposure or lifestyle factors. Some examples of how environmental epigenetics contribute to phenotypic alterations - the differences between worker and queen honeybees is brought about by the nutritional content of royal jelly which has been shown to be a consequence of a down-regulation of DNA methyltransferase during a critical period of development; an epimutation in humans where DNA methylation silences one allele of *MLH1* (DNA mismatch repair gene) has been demonstrated in a hereditary colorectal cancer (Daxinger and Whitelaw 2010). Epigenetic changes as a result of famine were evaluated in a cohort who'd endured starvation during the Dutch famine of 1944. Schizophrenia, obesity and cardiovascular diseases in later life were linked to under- nutrition at certain stages of pregnancy and it is postulated that they can be attributed to alteration in epigenetic programming ((Roseboom et al., 2006). It was evident that the stage of pregnancy in which famine occurred was critical to the observations. More recently it was demonstrated that epigenetic changes (histone modification) induced by the administration of CCl_4 to male rats conferred an adaptive healing response in the livers of F1 and F2 offspring (Zeybel et al 2012)

6) Recently attempts have been made to use epigenetic theory to elucidate neo-lamarckian evolutionary concepts. These are based on the premise that environmental factors can directly alter phenotype, often referred to as soft inheritance, which is independent of classical Darwinian theory of natural selection which requires a genetic mutation (Daxinger and Whitelaw 2010). In recent years an understanding of epigenetic changes which have the potential to be imprinted across generations has provided a possible molecular mechanistic basis for the lamarckian hypothesis (Skinner 2015). More broadly, it is hypothesised that transgenerational inheritance of germ cell epimutations are considered to behave like imprinted genes, managing to avoid the erasure and reprogramming events but this has not been directly demonstrated (Skinner et al 2015)

7) The role of methylation in transgenerational epigenetics was described briefly in the MUT/06/15. Since then, mechanistic theory has developed and there is much focus on the two waves of reprogramming which occur during post-fertilization and primordial germ cell (PGC) development. For an effect to be truly transgenerational (ie persist until F3 – the first unexposed generation) it means that the environmentally induced epimutations are in some way protected from reversal (Heard and Martienssen 2014).

8) The noncoding RNA (ncRNA) superfamily is considered to have an important regulatory role in epigenetic transgenerational transmission (Marczlo et al in press). It is suggested that ncRNA's impact on epigenetics in multiple ways including direct mechanisms such as the DNA methylation [e.g of *H19*, a paternally imprinted gene], histone and chromatin modelling. Furthermore, indirect mechanisms, such as the ability of ncRNA's to affect translation and transcription by impacting on mRNA stability may also contribute to alterations in the epigenome. For example, a mechanism by which epimutations avoid the global de- and re-methylation processes is postulated to involve sperm ncRNA's acting as templates or sequence guides, to reintroduce the epimutations into the oocytes during the fertilization, similar to those imprinted previously (Yan et al 2014). These properties are likened to those seen in imprinted genes. Whilst investigations of ncRNA alterations in relation to chemical induced transgenerational epigenetic changes are not as far advanced as those studying methylation changes, it is clear that a greater understanding of these processes will facilitate the elucidation of transgenerational epigenetic inheritance generally.

Phenotypic changes induced by Vinclozolin

9) Phenotypic changes considered to be a consequence of epigenetic mechanisms include; a decrease in fertility (decreased sperm counts), testes and prostate abnormalities, altered mate selection, ovarian cysts, decreased oocyte development, altered puberty onset (anogenital distance AGD), hypospadias (Nilsson and Skinner 2015). Many endocrine disruptors are thought to moderate the epigenome and that this contributes to transgenerational effects - one hypothesis is that this is mediated through steroid hormone receptors (Casati et la 2015).

10) There are a number of studies detailing the reproductive toxicity of Vinclozolin. The lowest NOAEL from reproduction studies evaluated by WHO is 4mg/kg/day (oral) based on reduced epididymal weight and reduction of fertility in F1 generation (2-generation reproduction toxicity study in rats) (WHO 1995). The EPA NOAEL used to derive the oral reference dose (RfD) is 2.5 mg/kg/day and is not based on reproductive toxicity endpoints but on organ weight changes in dogs (EPA[2]). A study which examined a narrow window of exposure (embryonic day (ED) 14 – post natal day PND3) reported findings such as reduction in AGD and retention of nipples in male pups and significant renal effects in offspring (Gray et al 1994). Another study which aimed to determine a precise period of sensitivity of mice to the anti-anandrogenic, reproductive effects of VZ used a very high dose (400mg/kg/day *orally*) determined ED 16-17 to be the most critical period for effects. Severe effects were present and 100% of male offspring were affected following administration of VZ to dams from ED14 -19. Effects included dose-related incidences and severities of male

[2] https://cfpub.epa.gov/ncea/iris/search/index.cfm?keyword=vinclozolin

reproductive tract malformations, retained nipples and reduced weight of sexual accessory glands. They report no changes when dams were dosed on ED12-13 (Wolf et al 2000).

11) Despite reports indicating limited changes following dosing at the earlier exposure window, the Skinner group focussed their attention on ED8-14, which they state is the period of gonadal sex determination and testis development in the rat. In a preliminary study by the Skinner group, exposure to 100mg/kg/day ip on ED8-14 caused testicular changes in offspring, principally testicular apoptosis (Uzumu et al 2004). The group consider this period to be more appropriate to study the effects of chemicals on testis rather than the later stage (ED 16-19) when reproductive tract abnormalities were seen. Therefore ED8-14 was selected as the exposure window for the series of studies investigating transgenerational epigenetic changes. The original Anway study (2005) reported increased sperm apoptosis, significantly reduced sperm counts and motility in F1-F3generations and >90% of males were affected. Additionally, 8% of males developed complete infertility associated with a lack of spermatogenesis and abnormal seminiferous tubule morphology. In an outcross experiment, VZ F2 males were mated with wild-type untreated females. The male progeny also had decreased sperm parameters.

Studies from the Skinner laboratory

12) An extensive range of studies published by the Skinner laboratories, were designed to examine their overarching hypothesis that environmental exposure inducing epigenetic changes can cause adult-onset reproductive diseases and infertility. They also aimed to test the hypothesis that epigenetic reprogramming of the male germ line enables the disease phenotype to become transgenerational so also examined methylation patterns, gene expression changes, including those that have a role in maintaining methylation. The use of the outcross lines investigates the importance of the male line in transferring the epigenetic changes.

13) The studies all use the same dosing regimen as used in the original Anway 2005 study. This is daily intraperitoneal (ip) dose of VZ at 100 mg/kg on ED 8-14 to F0 mothers. This dose was selected based on the results of the study in which rats exposed orally to 100 or 200mg/kg/day VZ between (ED)14 and postnatal day (PND)3 were observed to have external genitalia abnormalities (Gray et al 1994). However, it is not entirely clear why the ip route of administration was selected and not the oral route. The breeding protocol appears to be standardized throughout - as follows: Adult (PND60) F1 VZ-generation (offspring from F0 mothers) males were bred to F1 VZ-generation females from different litters to generate the F2 VZ generation. Adult F2 VZ-generation males were bred to F2 VZ-generation females to generate the F3 VZ-generation. Some studies also generated an F4 generation. Rats for the control (vehicle treated) groups (i.e., generations F1–F3) were bred in the same manner for all the generations. In some studies they also generated a VZ outcross (VOC) group when F2 generation VZ males were bred with wild-type females and a reverse outcross group (RVOC) when F2 VZ lineage females were bred to wild-type males. The use of these groups examined whether the effects were of male lineage. All studies utilised outbred Sprague Dawley rats (SD); some also included inbred Fischer rats and one study examined the effects in mice. Any discrepancies from this and when the VOC and RVOC lineages were used are indicated in Table 1.

14) The studies, in general, examined a different endpoint in each study – it is possible (likely?) that some of data have been generated from the same animals although it is not explicitly stated within each publication if this is the case. It is not always possible to determine the exact number of animals used in each study – where this was evident, the numbers are stated in Table 1. Many studies used testicular cell apoptosis as a marker of damage and the anti-androgenic effects of VZ. All studies report some transgenerational effect.

15) The first follow-up study examined the impact of VZ on spermatogenesis, involved analysis of different sperm stages (I-V; VI-V111; IX-XIV) and compared inbred and outbred strains of rat (Anway et al 2006a). The induction of testicular apoptosis was stage specific (IX-XIV). This was demonstrated in outcross groups (SD only) and in both rat strains although it was more marked in SD's (F1-F4). This study was followed up using techniques to examine whether the reported transgenerational effects (testicular apoptosis), and methylation changes resulted in or were the consequence of, changes to gene expression profiles in the testis (testis transcriptome) (Anway et al 2008a). Fetuses taken at ED16 (significance of this time point not clear) exhibited altered gene expression patterns across different generations. It was noted that 165 of the 196 genes which were altered across all generations were also seen in the VOC animal transcriptome. The authors examined the significance of the methyltransferase changes (Dnmt's) and used semi-quantitative PCR to confirm the microarray data. Dnmt3A and Dnmt3L were most notably altered - relative expression decreased through generations such that the relative expression in F3 generation was low.

16) A study examining only F1 generation animals (at ED13, 14 and 16) but which used the same species, strain, dosing protocol and microarray methodology as Anway et al (2008a) revealed 124 transcripts common to both studies and some of which were considered to be associated with the same epigenetic functions (Clement et al 2010). A study investigating the effects of VZ on prostate development and gene expression in the prostate revealed phenotypic (prostate epithelial cell atrophy) changes and identified altered cellular processes in F3 generation males (Anway and Skinner 2008b). Methylation status was not examined in this study.

17) Two studies examined the impact of VZ on female offspring endpoints and demonstrated transgenerational effects on pregnancy abnormalities and adult onset disease (eg ovarian cysts; Nilsson et al 2008, 2012). In the later study methylation and microarray data showed no overlap between the altered DMRs between ooctye and sperm suggesting that the epigenomes of different cell types are distinct. The authors suggest that transgenerational effects impacting the epigenome and the transcriptome of oocytes and granulosma cells may contribute to the development of ovarian disease.

18) Sperm epigenome changes following VZ administration to F0 generation females were examined in rats and mice by the Skinner group (Guerrero-Bosagna et al 2010, 2012 ANNEX 1). However it is of note that the protocols for investigating the methylation patterns, whilst similar, were not directly comparable. Changes in the F3 generation wre noted in both species suggesting that any epigenetic modifications have not been erased during embryonic development or germline reprogramming. In the rat study, focus was on examining genome wide promoter region patterns of methylation in F3 generation males. It is suggested that allelic differences in DNA methylation is characteristic of imprinted genes which are defined during the developmental period of the germ

line. The authors speculate on the significance of the promoter regions with altered methylation and the potential impact of the roles of the genes affected. . For example, olfactory receptor genes were identified in a couple of studies and the authors correlate this with data which demonstrates changes in mate selection following VZ exposure (Crews et al 2007). However this relationship does appear to be somewhat tenuous. 19) Transgenerational effects in both strains of mice were demonstrated across generations including testicular apoptosis as in the rat (Guerroro-Bosagna et al 2012). However adult onset disease phenotypes were apparent only in outbred CD1 mice And there was no overlap in methylation sites compared to the similar rat study.

20) Sertoli cells were the target examined in a further study using the same protocol (Guerrero-Bosagna et al 2013) with a view to evaluating the possible role of epigenetic changes in this potential target for transgenerational infertility. The authors discuss the possibility that transgenerational effects impacting the Sertoli cells could promote adult onset testis disease and Sertoli cell dysfunction. Testes apoptosis was examined as in the other studies but it is noted that the microarray and pathways analysis methods utilised were different from those used in previous studies. This study demonstrated only small increases in apoptosis in F3 generation The low density of CpG seen in sperm differentially methylated regions (DMR) was also seen in Sertoli DMR. It was considered that the majority of the differentially expressed genes did not contain a DMR which impacted on the regulation of gene expression. The authors make an association between the gene expression changes in pyruvate and lactate production pathways and VZ's effects on spermatogenesis but this appears to be speculation.

21) A key study examined methylation in PGCs isolated from embryonic testes at E13 and E16 (Skinner et al 2013). Interestingly it was reported that differential gene expression and methylation profiles in PGC's from F3 VZ treated lines were dissimilar between E13 and E16 and it was suggested that the differences were because E13 represents an epigenetic ground state, following maximal methylation erasure and E16 the re-methylated state. It was noted that DMRs identified exhibited similar features to those described in F3 sperm. A number of studies from this group identified a common DNA sequence motif , named 'Environmental Induced Differential Methylation Consensus Sequence 1' (EDM1) (Guerrero-Bosagna et al 2010, 2012; Skinner et al 2013) and the authors speculate that this genomic feature may be of importance in transgenerational programming. The motif was not identified in the Sertoli cell epigenome (Guerrero-Bosagna et al 2013). The motif identified in 75% of the confirmed promoters, was also present in 58.7% of imprinted gene promoter regions in the rat sperm but the significance of this was not evident. A more recent study investigated the possibility that epigenetic changes induced by VZ translate to genetic changes (Skinner et al 2015). – it was suggested that the transgenerational methylation changes in the sperm of F3 offspring promotes genomic instability as demonstrated by the increase in copy number variants (CNV).

22) Taguchi et al (2015) utilised principal component analysis (PCA) of the data generated by Skinner et al (2013). The technique described, PCA-based unsupervised feature examination (FE), identifies circulating mRNA's [and was developed with the aim of establishing specific blood-based biomarkers for disease (Taguchi and Murakami 2013 – in which combinations of specific mRNA's discriminate healthy controls from diseased human patients]. The method has been used previously to examine genotypes and DNA methylation profiles in cancer genetics to examine the correlations between DMR's and differentially expressed genes (Kinoshita 2014). In this study, the

differentially expressed genes and DNA methylation patterns between E13 and E16 were examined. The authors claim that enrichment analysis identified genes associated with aberrant promoter methylation which could be related to previously reported VZ induced effects in F3 although these assertions appear speculative. Chemokine signalling pathway genes and LRR proteins were highlighted as being of particular significance.

Studies from other laboratories

23) A number of studies set out to test the findings of the Anway et al (2005) and their claims of epigenetic mediated transgenerational effects following VZ exposure. The first study which examined transgenerational effects was published by Schneider et al (2008) and reported no transgenerational effects. Whilst this study did not measure any epigenetic endpoints, it evaluated the same phenotypic endpoints of the male reproductive system and is often cited as a key study that did not reproduce Anway's effects. It is noteworthy that they used oral rather than ip administration and Wistar, not SD rats. Their justification of the use of the oral route is that effects were seen in male offspring in a previous reproductive toxicity study at a much lower dose (3.125 mg/kg/day). The other notable difference is that the F1 males were mated with nulliparous females from an untreated lineage – and not F1 females from the VZ lineage. F1 % abnormal sperm were statistically higher in F2 and F3 treated males but this change was attributed to individuals with high variance. Apoptotic germ cell counts were reported as statistically lower in all generations. This is different from the Skinner studies which report increases. This effect was not considered to be treatment related by the authors as it was judged that anti-androgenic effects would increase apoptosis. The control apoptosis values in this study were highly variable. Spermatogenesis in male offspring of treated females (ie those exposed *in utero*) were also not affected and this was considered to be an anomaly. It was thought that the window of sensitivity for antiandrogenic effects is GD 16-20 as reported by Wolf (et al 2000) and Uzumu et al (2004).. This study did not replicate the Anway 2005 study for several reasons and it is also noteworthy that expected results (effects on sperm parameters in F1 males, exposed to VZ *in utero*) were also not observed.

24) The follow up study by the same group utilised the ip route of administration, and it appears that they also mated F1 males with F1 females and mated generations out until F4 (Schneider et al 2013). Again only reproductive parameters and testicular apoptosis were assessed. In this study, some increases in testicular apoptosis were demonstrated – the authors discuss that this finding is an expected outcome of anti-androgenic activity of VZ and argue that a methylation epigenetic mechanism for this effect is not conclusive. This argument was based on findings from a study that demonstrated *in utero* VZ -induced male reproductive tract abnormalities are mediated via the androgen receptor and that the mechanism was independent of Dnmt expression.

25) The study that most clearly contradicts the results of Anway et al was published by Inawaka et al (2009; ANNEX 1). They also used SD rats although they were sourced differently from the Skinner group studies (Crl:CD compared to Harlan Hsd?) and the same dosing protocol (100mg/kg// day ip for GD8-15) and subsequent generations were bred to F2. The initial F0 group sizes were small (n=4-5), although 50 F2 litters were examined. The authors report no phenotypic changes, including alterations in spermatogenesis and fertility/reproductive performance parameters. The

testicular cell apoptosis measurements were only reported from F1 generation VZ animals and it is noted that the standard deviation from the F0 animals was very large. However there appears to be a decrease in apoptosis by up to 50% in F1 treated lineage males. There were no changes to methylation patterns in sperm from F1 and F2 VZ generations compared to controls. The methodology used to examine methylation status was different from that used by Skinner/Anway group (a bisulfite sequencing technique as opposed to the immunoprecipitation method). However it is considered that both are sensitive and are commonly used for establishing methylation status in studies such as these.

26) Stouder et al (2010 ANNEX 1), established altered methylation patterns in imprinted genes from the sperm of mice in F1-F3 generations following treatment with VZ (50 mg/kg/day ip 10-18). A bisulphite technique was used to assess methylation. This study crossed F1 males from VZ lines with wild-type females to generate F2 and F3. Reduced methylation was identified in paternally imprinted genes *H19* and *Gtl2* and increased methylation in maternally imprinted genes *Peg1, Snrpn, Peg3*. The methylation changes diminished over generations and were not different from control in 3 of the 5 regions analysed in the F3 generation. Methylation changes were not detected in the genes from preparations of skeletal muscle DNA. They suggest that VZ induced methylation alterations and the imprinted status of these genes gives rise to the phenotypic transgenerational changes.

27) A recent study examined in more detail the methylation changes induced by endocrine disruptors including VZ and subsequent reprogramming (Iqbal et al 2015). A number of transgenic mouse lines dosed orally with 100mg/kg/day from ED8.5-13.5. The strain which contains a transgene enabled purification of the GFP-positive male and female germ cells and the JF1 inbred strain allowed measurement of allele specific transcription of known imprinted genes. It was concluded that although methylation events occurred that there was no evidence for an effect on methylation imprint erasure and hence persistent changes in subsequent generations. However it is noteworthy that no phenotypic endpoints were examined in this study (eg testicular apoptosis) and the strains of mice used were not the same as that used by the Skinner group.

28) Another recent study focussed on the role of miRNA's in mediating transgenerational effects induced by endocrine disruptors including VZ (Brieno-Enriquez et al 2015 –ANNEX 1). Specific miRNA's, considered to be key to modulating gene expressions critical to the development of germ cell differentiation, were examined in F1, F2 and F3 generation micebred from F0 animals treated with VZ in drinking water (doses of 1 and 100 mg/kg/day) for ED0.5-13.5. Testicular apoptosis was measured in adult testis and was statistically increased in both 1 and 100mg/kg/day groups in F1-F3 generations but increased only in F1 generation in the ED13.5 testes. SSEA-1 a marker of PGC number was reduced in F1 and F2 generations only, with no impact on F3. The regulation of the miRNA selected *Blimp1, Lin28 and let-7* are tightly correlated to ensure correct specification during embryo PGC development. Significant changes in the expression of a number of miRNA's were noted and a large increase in LIN28 protein in F3 compared to F1 and F2 generations was observed (in the absence of miRNA change). However, the methylation profiles of the genes considered to be of importance in PGC development were not altered. The authors conclude that VZ does not interfere with the process of methylation reprogramming at ED13.5 and suggested a role for miRNA's in paternal transgenerational transmission.

Discussion:

29) Historically, reproductive toxicity studies demonstrated effects of VZ on F1 generation animals (offspring exposed *in utero*) and it was hypothesized that the observations were attributable to VZ binding with androgen receptors (Gray et al 1994). The Anway study (2005) introduced the possibility that the effects persist to subsequent generations F2 and F3, thus an indirect mechanism was sought. Alterations in DNA methylation patterns provided a plausible mechanism by which a true transgenerational effect could manifest and preliminary investigations of methylation status were reported in this original study. The studies suggest that there is a critical window of exposure for the induction of transgenerational effects and that coincides with the later stages of PGC migration and the colonization of foetal gonads. The importance of germline DNA demethylation – remethylation is highlighted although a mechanism by which this process is bypassed remains elusive. It was postulated that the decreases of the Dnmt's observed could contribute to establishing the altered epigenome enabling it to become transgenerational. Histone modification and the importance of mirna's are also implicated in the mechanism (Anway 2006)

27) All subsequent studies from the Skinner laboratory report positive findings. The endpoints examined include: testes, sertoli cells, primordial germ cells, the prostate, adult onset disease in male and female offspring – however not all studies examine phenotypic endpoints, methylation and gene expression changes and it is possible that publication bias may skew the reporting of the various endpoints. The dose utilised is very high – an ip dose is not representative of a potential route of exposure in man and is likely to provide a model for hazard identification at best. Alyea et al (2014) Dow Chemical Company provide a risk assessment of VZ based on the epigenetic endpoints. It is pointed out that the dose used by Skinner to induce the transgenerational effects identified (100 mg/kg/day ip) is 80-fold greater than the lowest NOEL from an oral rat study (1.2 mg/kg/day) and therefore 1,200,000 fold greater than human exposure (based on a RfD of 0.0012 mg/kg/day).

27) The studies by Schneider et al (2008, 2013) which discount the phenotypic transgenerational effects have several significant protocol differences to the Anway study (and Skinner general protocol) and also have not demonstrated expected anti-androgen effects. Other studies have confirmed a transgenerational effect for VZ in rats and there is a study which postulates that the mechanism by which the PGC escape methylation erasure may be mediated by miRNA's (Brieno_Enriquez et al 2015). However, no transgenerational effects were demonstrated by Inawaka (2009) and this is supported by a study claiming that epigenetic effects induced by VZ are corrected by epigenome reprogramming (Iqbal et al 2015). To date there are no studies which have aimed to examine dose responses. Furthermore it is likely that factors such as species or strain differences, exposure windows are critical to understanding the patterns of epigenetic change.

28) LeBaron et al (2010) examined epigenetics within the context of 'traditional' toxicity assays and analysed the data available on diestylstibestrol, bisphenol A and VZ. They concluded that the current testing strategy is able to detect adverse effects of chemicals regardless of the mechanism, including epigenetic changes. The VZ studies may indicate that there is significant protection against epigenetic changes compared to other endpoints.

29) There are a number of European and international initiatives aiming to address the questions surrounding the relevance of epigenetics in toxicology and examining the approaches that can be used to identify and quantify epigenetic changes. A synopsis of an ILSI/HESI symposium entitled 'advances in assessing adverse epigenetic effects of drugs and chemicals' held in November 2013 has been published (Miousse et al 2015). The overall conclusions were that significant developments in methodologies for assessing epigenetic endpoints and that it is plausible to address this in safety assessment paradigms. The challenge of understanding the natural variability between strains, species, sex and age and what constitutes a 'healthy' or 'diseased' was recognised.

30) An ECETOC workshop report from 2011 concluded that demonstration of epigenetic changes does not establish cause and effect and that it was important to investigative the relevance of any changes. http://www.ecetoc.org/publications/workshop-reports/ Further ECETOC workshops have taken place 'the role of epigenetics in reproductive toxicity' (November 2015) and 'non-coding RNA's and risk assessment science' March 2016. An EFSA scientific colloquium entitled 'Epigenetics and Risk Assessment: Where do we stand?' is scheduled to take place in June 2016.

Secretariat/PHE Toxicology Unit May 2016

References:

Alyea RA, Gollapudi BB, Rasoulpour RJ. (2014) Are we ready to consider transgenerational epigenetic effects in human health **risk assessment?** Environ Mol Mutagen. ;55(3):292-8

Anway MD, Cupp AS, Uzumcu M, Skinner MK. (2005) Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science. 3;308(5727):1466-9.

Anway MD, Memon MA, Uzumcu M, Skinner MK. (2006a) Transgenerational effect of the endocrine disruptor vinclozolin on male spermatogenesis. J Androl. 27(6):868-79

Anway MD, Leathers C, Skinner MK. (2006b) Endocrine disruptor vinclozolin induced epigenetic transgenerational adult-onset disease. Endocrinology. 147(12):5515-23.

Anway MD, Rekow SS, Skinner MK. (2008a) Transgenerational epigenetic programming of the embryonic testis transcriptome. Genomics. ;91.:30-40

Anway MD, Skinner MK. (2008b) Transgenerational effects of the endocrine disruptor vinclozolin on the prostate transcriptome and adult onset disease. Prostate. 2008 Apr 1;68(5):517-29.

Brieño-Enríquez MA, García-López J, et al 2015 Exposure to endocrine disruptor induces transgenerational epigenetic deregulation of microRNAs in primordial germ cells. PLoS One. 21;10(4):e0124296.

Casati L, Sendra R, Sibilia V, Celotti F. (2015) Endocrine disrupters: the new players able to affect the epigenome. Front Cell Dev Biol. 18;3:37.

Clement TM., Savenkova MI, Settles M, Anway MD, Skinner MK. (2010) Alterations in the developing testis transcriptome following embryonic vinclozolin exposure. Reprod Toxicol.;30(3):353-64.

Cowin PA., Gold E, Aleksova J, O'Bryan MK, Foster PM, Scott HS, Risbridger GP. (2010) Vinclozolin exposure in utero induces postpubertal prostatitis and reduces sperm production via a reversible hormone-regulated mechanism. Endocrinology. 151.:783-92

Daxinger L., Whitelaw E. (2010) Transgenerational epigenetic inheritance: more questions than answers. Genome Res. 20(12):1623-8.

Gray LE Jr., Ostby JS, Kelce WR. (1994) Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex D ifferentiation of the male rat. Toxicol Appl Pharmacol. ;129.:46-52.

Guerrero-Bosagna C., Settles M, Lucker B, Skinner MK. (2010) Epigenetic transgenerational actions of vinclozolin on promoter regions of the sperm epigenome. PLoS One. 30;5(9). pii: e13100.

Guerrero-Bosagna C., Covert TR, Haque MM, Settles M, Nilsson EE, Anway MD,Skinner MK. Epigenetic transgenerational inheritance of vinclozolin induced mouse adult onset disease and associated sperm epigenome biomarkers. Reprod Toxicol. ;34(4):694-707.

Guerrero-Bosagna C., Savenkova M, Haque MM, Nilsson E, Skinner MK. (2013) Environmentally induced epigenetic transgenerational inheritance of altered Sertoli cell transcriptome and epigenome: molecular etiology of male infertility. PLoS One. ;8(3):e59922.

Heard E, Martienssen RA. (2014) Transgenerational epigenetic inheritance: myths and mechanisms. Cell. 27;157.:95-109.

Inawaka K, Kawabe M, Takahashi S, Doi Y, Tomigahara Y, Tarui H, Abe J, Kawamura S, Shirai T. (2009) Maternal exposure to anti-androgenic compounds, vinclozolin, flutamide and procymidone, has no effects on spermatogenesis and DNA methylation in male rats of subsequent generations. Toxicol Appl Pharmacol. ;237.:178-87.

Iqbal K, Tran DA, Li AX, Warden C, Bai AY, Singh P, Wu X, Pfeifer GP, Szabó PE. (2015) Deleterious effects of endocrine disruptors are corrected in the mammalian germline by epigenome reprogramming. Genome Biol. 27;16:59.

LeBaron MJ., Rasoulpour RJ, Klapacz J, Ellis-Hutchings RG, Hollnagel HM, Gollapudi BB. (2010) Epigenetics and chemical safety assessment. Mutat Res. 705.:83-95.

Manikkam M., Guerrero-Bosagna C, Tracey R, Haque MM, Skinner MK. (2012) Transgenerational actions of environmental compounds on reproductive disease and identification of epigenetic biomarkers of ancestral exposures. PLoS One.

Miousse IR, Currie R, Datta K, et al (2015) Importance of investigating epigenetic alterations for industry and regulators: An appraisal of current efforts by the Health and Environmental Sciences Institute. Toxicology. 335:11-9.

Nilsson EE., Anway MD, Stanfield J, Skinner MK. (2008) Transgenerational epigenetic effects of the endocrine disruptor vinclozolin on pregnancies and female adult onset disease. Reproduction.;135(5):713-21.

Nilsson E., Larsen G, Manikkam M, Guerrero-Bosagna C, Savenkova MI, Skinner MK. (2012) Environmentally induced epigenetic transgenerational inheritance of ovarian disease. PLoS One. ;7(5):e36129.

Roseboom, T., de Rooij, S., and Painter, R. (2006). The Dutch famine and its long-term consequences for adult health. Early human development *82*, 485-491.

Schneider S, Kaufmann W, Buesen R, van Ravenzwaay B. (2008) Vinclozolin--the lack of a transgenerational effect after oral maternal exposure during organogenesis. Reprod Toxicol. 25(3):352-60

Schneider S, Marxfeld H, Gröters S, Buesen R, van Ravenzwaay B. (2013) Vinclozolin--no transgenerational inheritance of anti-androgenic effects after maternal exposure during organogenesis via the intraperitoneal route. Reprod Toxicol. ;37:6-14.

Skinner MK, Guerrero-Bosagna C, Haque M, Nilsson E, Bhandari R, McCarrey JR. (2013) Environmentally induced transgenerational epigenetic reprogramming of primordial germ cells and the subsequent germ line. PLoS One. 15;8(7):e66318.

Skinner MK. (2015) Environmental Epigenetics and a Unified Theory of the Molecular Aspects of Evolution: A Neo-Lamarckian Concept that Facilitates Neo-Darwinian Evolution. Genome Biol Evol. 26;7(5):1296-302.

Stouder C, Paoloni-Giacobino A. (2010) Transgenerational effects of the endocrine disruptor vinclozolin on the methylation pattern of imprinted genes in the mouse sperm. Reproduction. 139.:373-9.

Taguchi YH. (2015) Identification of aberrant gene expression associated with aberrant promoter methylation in primordial germ cells between E13 and E16 rat F3 generation vinclozolin lineage. BMC Bioinformatics.;16 Suppl 18:S16.

Taguchi YH, Murakami Y. *(2013) Principal component analysis based feature extraction approach to identify circulating microRNA biomarkers. PLoS One. 24;8(6):

Uzumcu M., Suzuki H, Skinner MK. (2004) Effect of the anti-androgenic endocrine disruptor vinclozolin on embryonic testis cord formation and postnatal testis development and function. Reprod Toxicol. ;18(6):765-74.

van Otterdijk SD(, Michels KB. (2016) Transgenerational epigenetic inheritance in mammals: how good is the evidence? FASEB J. 1. pii: fj.201500083

Whitelaw E. Disputing Lamarckian epigenetic inheritance in mammals. Genome Biol.27;16:60.

Wolf CJ, LeBlanc GA, Ostby JS, Gray LE Jr. (2000) Characterization of the period of sensitivity of fetal male sexual development to vinclozolin. Toxicol Sci. 2000 May;55.:152-61.

Wolf CJ, LeBlanc GA, Ostby JS, Gray LE Jr. (2000) Characterization of the period of sensitivity of fetal male sexual development to vinclozolin. Toxicol Sci. ;55.:152-61.

Yan w, (2014) Potential roles of noncoding RNAs in environmental epigenetic transgeneration inheritance Mol. Cell Endocrinol. 398; 24-30

Zeybel M, Hardy T, Wong YK, et al (2012) Multigenerational epigenetic adaptation of the hepatic wound-healing response. Nat Med.18(9):1369-77.

ANNEX 2: Tabulated summaries of in vivo studies examining transgenerational epigenetic effects of maternal Vinclozolin exposure

ED = embryonic day : PND = postnatal day DMR = differentially methylated region
VZ outcross (VOC) group = F2 generation VZ lineage males with wildtype females Reverse outcross group (RVOC) = F2 generation VZ lineage females to wildtype males

TABLE 1: Studies conducted by Skinner laboratory (in chronological order)

Authors	Species	Dosing protocol	Breeding protocol	Tissues and endpoints	Results
Anway et al 2006a	Rats Sprague Dawley (SD) n= 4 Rats Fischer (CDF) n=3	Pregnant F0 females - VZ (in DMSO) ip at 100mg/kg/day for ED8-14 (replicate Anway 2005)	F1-F4 generation animals for SD and F1-F3 for Fischer derived from F0 VZ and control treated females VOC and RVOC	**Spermatogenesis** Sperm motility and concentration from PND60 males Testicular histology, including detailed sperm staging and apoptosis (TUNEL) from P60 Homogenization-resistant spermatid analysis	No effect on reproductive parameters in F1-F4. Slight decrease in number of pups in F1 Apoptotic sperm were increased in VZ lineage F1-F4 and VOC males at spermatogenesis stages IX-XIV and in F1 and F3 at stages I-V. In SD Increased in F1-F3 in Fischers (only bred to F3) in IX- XIV only. RVOC results were similar to the controls. -
Anway et al 2006b	Rats (SD) n≥6	Pregnant F0 females - VZ (in DMSO) ip at 100mg/kg/day for ED8-14	F1-F4 generations (bred at PND60) VOC RVOC	**Adult onset disease:** Males and females collected at 6-14 months for investigations of adult onset disease – pathology including testes, kidney, immune system,	Increase in tumours in VZ lineage F1-F4 animals compared to controls Prostatic lesions)(atrophy, prostattis) increased Renal lesions increased
Anway et al 2008a	Rats (SD) n= 8 (ie 8 x F0 females)	Pregnant F0 females - VZ (in DMSO + sesame oil) ip 100mg/kg/day for ED8-14 (replicate Anway 2005)	F1-F3 generation animals derived from F0 VZ and control treated females – VOC group F1 RVOC	**Testes** from offspring of all generation taken at ED16. Histology n=6 from three different lines for each generation Apoptosis in testes using TUNEL Microarray – RNA from 3 different lines (18-25 male pups) pooled. Affymetrix Rat 230 2.0 gene chip (30,000 transcripts). Two different experiments conducted Semiquantitiative PCR was performed on	No histological changes Increased apoptosis in VZ lineage offspring in F1 and F2 . Compared to controls F1 VZ testes had 2071 altered genes; F2 1375; F3 566 (90% of these were decreases). 196 genes common to all generations. 165 of these were also seen in VOC group. Cluster analysis showed categories strongly represented included transcriptional regulation, signal transduction, cytoskeleton. Relative expression of methyltransferases

				some methyltransfereases to confirm microarray data.	Dnmt1, 2, 3A, 3B, 3L. – all decreased – most notably 3A (in F1 and F2) and 3L (in all three). Some genes associated with histone modification were also highlighted.
Anway and Skinner 2008b	Rats (SD) n=7 for controls and VZ	Pregnant F0 females - VZ (in DMSO) ip at 100mg/kg/day for ED8-14	As Anway 2006 to F4 VOC and RVOC	**Prostate** examinations on F1-F4 generations at PND 15, 30, 45, 70-120-and 180-300 (min of 3 litters form each generation – variable numbers of control and VZ – n=10-39) – histology and apoptosis (TUNEL). Prostate epithelial cells were isolated from PND45 males – RNA isolated from isolated cells and from PND180 males from whole ventrial prostate from F3 Microarray – prostate RNA hybridized to Affymetric rat 230 2.0 chip. Biotinylated RNA visualised by labelling. Serum testosterone	No effect on reproductive parameters in any generation. Prostate abnormalities – ventral epithelial cell atrophy in F1-F4 VZ generations at approx 30%. Increased in adults (ie PND 160) and reduced ventral prostate/body weight ratio. Decreased testosterone in VOC males. 954 genes altered between control and VZ F3 males (~50% increased in expression) 259 in isolated epithelial cells – (age?) >Functional pathways identified include transcription, metabolism, immune response growth factors 7 upregulated genes. 55 common between whole prostate and epithelial cell preparations – signalling pathways – genes associated with prostate disease highlighted.
Nilsson et al 2008	Rats (SD) n=6	Pregnant F0 females - VZ (in DMSO) ip at 100mg/kg/day for ED8-14	F1-F3 generation animals derived from F0 VZ and control treated females – (F1 n=53; F2 n= 41; F3 n= 11_ VOC, RVOC	**Female adult onset disease** Haematology and biochemistry Pathology	9/105 rats (8.6%) from the VZ F1-F3 lineage exhibited uterine haemorrhage &/or anaemia late in pregnancy compared to 0 in controls Complete blood cell counts and serum chemistry profiles demonstrated that selected VZ animals lineage animals, but not controls - marked regenerative anaemia in late pregnancy. Kidney histology -67% glomerular abnormalities in VZ F2 and F3 compared with 18% of the controls. VZ generation animals also developed various types of tumours in 6.5% of animals compared to 2% of control

Authors	Species	Dosing protocol	Breeding protocol	Tissues and endpoints	Results
Clement et al 2010	Rats (SD) n = not given	Pregnant females dosed with VZ (in DMSO) ip at 100mg/kg/day (GD) E8-14 0r vehicle	**F1 only** - pups taken on ED13, 14 and 16 Embryo sex was determined identified using specific primers (Sry)	**Embryonic gonads** were cultured for 3 days - the pairs were split - one treated with VZ at 50μM and one control. Histology RNA extracted from one – biotin labelled Microarray –hybridized to rat RAE230 2.0 arrays containing 31,099 transcripts (Affymetrix). Array signals calculated using RMA, GCRMA, PLIER and MAS5. KEGG analysis	No histological differences between VZ and control gonads at ED13, 14 and 16 (period of gonad differentiation) Microarray – significant changes (≥1.5-fold) from VZ vs controls - 576 altered genes from E13, E14 and E16 combined although most only altered at only one time points, 26 at 2 timepoints and none at all 3 timepoints. Identified pathways included = calcium signalling, transcription signalling, protein modification, epigenetics, development. Candidate genes for VZ action on differentiating testis from these pathways noted. Differences between in vitro treatment with VZ vs control – 19 altered transcripts – 3 of which were amongst the 576 altered following in vivo treatment
Guerrero-Bosagna et al 2010	Rats (SD) 8 lines (ie 8 x F0 females)	Pregnant F0 females - VZ (in DMSO) ip at 100mg/kg/day for ED8-15	F1-F3 generation animals derived from F0 control and VZ treated females – all others controls . Two experimental groups a year apart = V1/C1 and V2/C2	**Sperm** DNA extracted from 6x F3 VZ and control (3 from each experiment) and pooled to give 4 groups (V1, V2, C1 & C2). Each pooled sample - Immunoprecipitation of methylated DNA fragments (MeDIP) - Tiling microarray – MeDIP Chip analysis - methyl-cytosine antibody promoter Hybridization to Rat ChIP 385K Promoter 2 array set – two comparisons of treated vs controls Individual CpG methylation analyses .	52 differential methylation regions in VZ samples compared to control – the patterns were seen within 48 different promoters of the sperm epigenome. 16 of these regions were analysed. Consistency was observed between the individual and pooled samples. Common sequence motifs were identified – EDM1 –in 75% of identified promoter regions this motif was not identified in other random areas of the promoter regions (those without methylation changes). An altered CNV was described in a candidate promoter region - Fam111a –

Authors	Species	Dosing protocol	Breeding protocol	Tissues and endpoints	Results
Guerrero-Bosagna et al 2012	Inbred 129 **mice** CD1 mice (outbred)	Pregnant F0 females - VZ (DMSO in sesame oil) ip at 100mg/kg/day for ED7-13	F1-F3 generations derived from F0 control and VZ treated females crossed with PND60 -90 and 1 year males from F1-F3 generation used for pathology and apoptosis	**Sperm** motility analysis Testes pathology Testicular apoptosis – TUNEL Ovarian cysts All from both strains **F3 generation examined only (PND 60-90)** Sperm DNA isolated from F3 (CD-1 only) – processed for methylated DNA immunoprecipitation (MeDIP) and array MeDIP-Chip analysis (Affymetrix Gene Chip Mouse Promoter 1.0R array) Quantitative PCR methylation (same as G-B 2010)	No effects on reproductive parameters Apoptotic germ cells – increased in F1-F3 generations at PND60-90 and at 1 year in both mouse strains. Sperm motility - reduction only in F1 generation. Ovarian cysts: significantly increased in F1, F2 & F3 generations Strain differences were seen – no changes to testis, prostate or kidney pathology in 129 mice Sperm epigenome changes Stat sig different DMR, (increased and decreased), in 66 different promoters – many but not all, changes confirmed by PCR. EDM1 motif identified in DMR and demonstrated to be similar to the rat. However there were no overlap of the methylation sites
Guerrero-Bosagna et al 2013	Rats (SD) 8 lines (ie 8 x F0 females)	Pregnant F0 females - VZ (in DMSO) ip at 100mg/kg/day for ED8-15 (replicate Anway 2005) + equal volume of sesame oil	F1-F3 generation animals derived from F0 control or VZ treated females –	Testes apoptosis –TUNEL from F1 and F3 generations at 1 year of age **Sertoli cells** prepared from PND20 F3 males. RNA was extracted and hybridized to Rat Gene 1.0 ST microarray (27,000 transcripts – Differentially expressed genes were examined using KEGG Pathway and Pathway express gene network analyses. Extracted DNA and methylated DNA immunoprecipitation (MeDIP) to identify significant changes in DNA methylation Tiling microarray – MeDIP Chip analysis -	Slight increases in sperm apoptosis Sertoli cell transcriptome changes - identified 417 differential expressed genes in VZ F3 compared to controls – 22 pathways identified - some key enzymes in pyruvate and lactate metabolism pathway were downregulated and highlighted by authors oxidative phosphorylation, signal transduction . Transgenerational epigenome of sertoli cells - 101 DMR changes although none of these areas were common to those previously

					identified in sperm . The previously induced DNA sequence motif EDM1 was not stat sig different from control
Authors	Species	Dosing protocol	Breeding protocol	Tissues and endpoints	Results
Nilson et al 2012	Rats (SD) 8 lines (ie 8 x F0 females)????	Pregnant F0 females - VZ (in DMSO) ip at 100mg/kg/day for ED8-14 (	F1-F3 generation animals derived from F0 VZ and control treated females – all others controls	**Ovaries** taken from F1 and F3 generation animals at 12 months - examined histopathologically with particular attention to the number and size of cysts. F3 generation rats from VX and control lineages were treated with pregnant mare gonadotrophin at 5-6 months of age to identify if there were any changes in ovulation and granulosa cell development Microarray transcriptome analysis mRNA was hybridized to Affymetrix Scanner 3000 Epigenetic endpoints were also MeDIP as described previously and Tiling array MeDIP-chip analysis . Statistical analyses Neonatal (from day 4 rats) ovaries from untreated 4 day old rats were cultured , and exposed to vinclozalin at 50, 100, 200 or 500µM for 10 days (culture medium replaced every 2 days). These ovaries were examined histologically for ooctye number.	In F1 and F3 females there was a stat signif reduction in the number of follicles and increase in the number of small and large cysts following treatment with VZ indicating a transgenerational effect. The later effect was more pronounced in the F3 generation compared to the F1 generation. The authors identified differentially expressed genes and examined cellular pathways and processes impacted by these changes across generations in granulosa cells - transgenerational . Two pathways highlighted were PPAR signalling and steroid biosynthesis. Epigenetic investigations indicated 43 differential DMR's in F3 generation but there were 523 differentially expressed genes– therefore most differentially expressed genes did not have a DMR in their promoter region. Suggested that the gene expression change could be mediated via nc RNA's . The number of oocytes in cultured ovaries decreased dose dependently but was statistically different to control only at 500 µM.
Skinner et al 2013	Rats (SD) Hsd 8 lines (ie	Pregnant F0 females - VZ (in DMSO) ip at	F1-F3 generation animals derived from F0 VZ or control	**Foetal germ cell** preparations from embryo gonads taken on ED13 and ED16. RNA extracted was hybridised to Rat Gene 1.0 ST	At ED13 there were 592 differentially expressed genes (177 increased and 388 decreased) and 148 at ED16 (77 increased

	8 x F0 females)?	100mg/kg/day for ED8-14 + equal volume of sesame oil	treated females	microarray (Affimetrix) containing 27000 transcripts. Pathway gene network analysis –KEGG and functional relationships of differentially expressed genes from F3 generation–. DNA isolated - MeDIP and tilling array and MeDIP-Chip bioinformatics to determine methylation Reproducibility was assured when the candidate DMR showed significant changes in all three paired comparisons.	and 67 decreased). Pathway cluster analysis strongly revealed pathways associated with olfactory induction – 64 genes which require critical epigenetic regulation. There was negligible overlap between deregulated genes from the different embryo time-points but it is reported that they impact on several of the same pathways . 257 and 242 DMR's (epimutations) were identified in E13 and E16 PGC's respectively. The regions had similar features – low CpG density, increase in a rich DNA sequence motif similar to DNA methylation motif 1 (EDM1),
Skinner et al 2015	Rats (SD Hsd) 1st expt:	Pregnant F0 females - VZ (in DMSO) ip at 100mg/kg/day for ED8-14	To F3 Two experiments conducted epimutations nd CNV studies separately. N=6 for epigenetic evaluation at F3, n=18 for genetic analysis (9 xF1 and 9 x F3)	DNA isolated from epididymal sperm (from 9 F1 generation and 15 F3 generation) at PND120/. Immunoprecipitation MeDIP = genome wide analysis **CNV analysis** (samples distinct from Me analysis) – custom designed array whole genome tiling array 385,102 probes – from 3 individuals to generate 3 different DNA pools	9.932 DMR's identified using single oligonucleotide resolution and 191 using a >3 oligonucleotide resolution. – compared with the 52 promoter region DMR's identified in G-B 2010 overlap of 15 associated genes but not at the specifically identified DMR sites. CNV in F1 VZ lineage – 540 single probe sites and 39 >3 probe sites (3-adjacent probe sites - considered to be more reflective and less potential for false positives) – however these were consider to fall within random probability . F3 4912 single probe sites and 503 >3 probe sites. Cluster analysis indicated regions which may be more sensitive to epigenetic influence promoting CNV formation. No overlap between the epimutations (DMRs) and CNVs

Table 2: Studies conducted by laboratories other than the Skinner laboratory

Authors	Species	Dosing protocol	Breeding protocol	Tissues and endpoints	Results
Schneider et al 2008	Wistar rats (20/group)	Pregnant) F0 VZ orally at 0, 4 or 100 mg/kg/day on days GD6-15	F1 males mated with untreated females to generate F2 , the males of which were mated with untreated females to generate F3.	Standard reproductive parameters were examined (fertility indices, gestation, viability, lactation. Testicular apoptosis using TUNEL	No effects on reproductive parameters at F1-F3 Generation Increase in %males with abnormal sperm at 100mg/kg/ in F1-F3. Decrease in apoptotic sperm in F1-F3.
Inawaka et al 2009	Rats (SD Crl:CD) n=4 -5	Pregnant F0 females - VZ (in DMSO) ip at 100mg/kg/day for GD8-15 (replicate Anway 2005)	F0 females delivered and raised pups to weaning. F1 males mated with untreated females to produce F2.	Testes histopathology and methylation status F1 &F2 males for testes (sperm) methylation analysis at PND6 (n=1-2 from each litter) and 13 weeks (n=1). Testis histology and sperm parameters at 13 weeks (n=1). Necropsy on F0 and F1 males at 13 weeks and parental F1 males at 20 weeks. Methylation method –genomic sperm DNA extracted – methylation status analysed using bisulfite sequencing method (7x CpG sites within *LPlase* gene as described in Anway 2005. Methylation rate % = number of methylated closes /number all clones x 100	No abnormalities or alterations in spermatogenesis, fertility or DNA methylation in all generations examined (F0-F2) No transgenerational effects observed.

Authors	Species	Dosing protocol	Breeding protocol	Tissues and endpoints	Results
Stouder and Paolin-Giacobino 2010	Mice (FVB/N) N=5	Pregnant females dosed with VZ ip at 50mg/kg (in corn oil) ED10-18	F1 males bred with wildtype females to produce F2	Sprem collected at 2 months DNA extraction Methylation by bisulphite treatment (EZ gold kit Zymo) PCR amplification of differentially methylayed domains (DMDs)	VZ induced significant changes in methylation of CpG sites in sperm of all generations in two paternally (*H19* and *Gtl2 – reduced methylation*) and three maternally (*Peg1, Snrpn* and *Peg3 – increased methylation*) imprinted genes. No specific CpG more sensitive than others . Methylation changes not marked in livers or skeletal muscle
Schneider et al 2013	Rats (Wistar Crl:WI[Han }) 20/group	Pregnant females dosed with VZ ip at 0, 4 or 100 mg/kg/day fro (in sesame oil) at ED6-15	At least one M&F from each F1 litter mated – 1-2 from F2 and F3 litters to give 50 males which were mated with untreated F (are these from outside the VZ group?)	Traditional toxicity endpoints In life observations on all F1-F4 offspring. Sexual maturation from PP 40. At 127-134 days F1-F4 males were killed, sex organs examined , testes apoptosis, , standard reproductive parameters	One case of hypospospadias in F1 . Slightly increased time to preputial separation in F1 at 100mg/kg. Stat signif increases in testes apoptosis in F1 and F2 at 4mg/.kg and in F1m F2 and F3 of 100 mg/kg groups.
Brieno-Enriquez et al 2015	Mice CD-1 10/group fro F1 assessmen t 10/group for further breeding	Pregnant females dosed with VZ orally (drinking water) at 0, 1 or 100 mg/kg (dissolved in DMSO) for ED 0.5-13.5	25 F1 males bred with unexposed females to obtain F2, and then F3 25 litters from each generation taken and mated with control females to assess male fertility	Gonads taken from offspring of F1 foetuses Fertility assessment Testes histology, seminiferous tubule morphometry PGC's isolated from ED 13.5 males (from each generation?) - Testes c ell apoptosis (TUNEL) Histological analysis of embryonic testes (ED13.5) SSEA-1 as a marker of PGC number. Specific miRNA expression by RT-qPCR (candidates selected pri-let-7a, let-7a-1-3p, Lin28a, Lin28b– believed to have roles in germ cell development). Methylation analysis using a bisulphite sequencing technique (RRBS) on DNA prepared from PGC's –(libraries amplified by PCR paired-end sequencing on an Illumina	Fertility was reduced by 8% at 1mg/kg and 12% at 100mg/kg in F1 generation. This was associated with increase in seminiferous tubule damage Apoptosis in testes stat sig increase in adults in F1, F2 and F3 in 1 and 100 mg/kg groups – TUNEL positive signals increased in ED13.5 in F1 in 1 and 100 mg/kg and in F2 at 100mg/kg. SSEA-1 positive signals decreased at 1 and 100 at F1 and F2. At ED 13.5. = decrease in PGC in ED13.5 in F1 and F2 at 1` and 100mg/kg. Changes in miRNA's significantly different between doses at F1, F2 and F3. LIN28 protein expression greatly increased at F3 compared to F1 and F2 Methylation VZ did not alter the erasure of PGC

				HiSeq2000?)	methylation at ED 13.5. No changes in distribution of methylated CG's or selected miRNAs (lin28a, lin28b, Blimp)
Iqbal et al 2015	Mice Transgenic TgOG2 , inbred FVB 129S1, JF1	Pregnant females dosed with VZ orally 100mg/kg (in corn oil) from ED8.5-13.5.	?? to F3 (but not described in methods?) G1 males mated with unexposed Jf1 females = G2 A variety of different breeding protocols.	Foetal germ cells isolated from d9.5, 13.5 and 17.5 embryos –M&F sperated by FACS. DNA isolated and methylation status examined using MIRA (methylated CpG island recovery assay) and MIRA-chip. RNA isolated and hybridized to mouse Gene 1.0ST array (Affymetrix). cDNA reversed transcribed from RNA using rtPCR (Invitrogen).	Allele specific transcription patterns in control M&F germ cells from F1 for known imprinted genes demonstrated. VZ caused a decrease in transcription in *Nesp* and increase in *Meg3* in MGC. (erased imprinted baseline) but not considered to be significant. G1 129S1 male offspring – allell specific DNA methylation patterns at DMR's in somatic cells . G2 somatic cells CpG methylayed fraction using MIRA at 14 MR's (18SNP) From 378 different conditions (7 organs x 3 ED's x 18 SNP's) only 4 signif changes in methylation patterns (5-10% change) FGC from In utero exposed G1 FGC (from 129S1) mated with unexposed JF1 M to generate F2 offspring