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COMMISSION ON HUMAN MEDICINES AD HOC EXPERT GROUP

Title of paper: Evaluation of new research on the developmental effects of norethisterone acetate and ethinylestradiol in zebrafish embryos.

Type of paper: For advice

Product(s):	**Assessors:**
Primodos (no longer marketed) Hormonal contraceptives (numerous) Gynaecological products (numerous)	**Non-clinical assessors**: John Clements Ross Hawkes
Active constituents(s): Norethisterone acetate; Ethinylestradiol	**Previous Assessment(s):**
MAHs: *For Primodos and other HPTs*: Alinter Group Bayer plc GlaxoSmithKline UK Marshall's Pharmaceuticals Ltd Merck, Sharpe and Dohme Ltd Pfizer Piramal Healthcare Ltd Sanofi *For hormonal contraceptives and other gynaecological products*: numerous	**Legal status:** Primodos: not applicable
Therapeutic classification: POM	

Contents

1. Issue

Brown et al. published a study in Scientific Reports[1] on 13th February 2018 which concluded that the components of Primodos, norethisterone acetate (NETA) and ethinylestradiol (EE), induced developmental abnormalities in zebrafish embryos. NETA and EE historically were used in hormone pregnancy tests (HPTs) such as Primodos from late 1950s until 1978. Currently, NETA and EE are found in a range of widely-used authorised gynaecological medicines across the EU including oral contraceptives, hormone replacement therapies, treatments for endometriosis, disorders of menstruation, period delay and some cancers. Given the specialist nature of the study and that NETA and/or EE are found in currently authorised gynaecological medicines, the Commission on Human Medicines (CHM) advised that an ad hoc meeting of experts should be convened to carefully evaluate the new study and its findings.

2. Terms of reference for the review

The ad hoc Expert Group is asked to carefully evaluate the study by Brown et al. on the effects of NETA/EE on the development of zebrafish embryos and to advise CHM on:

- The suitability of the zebrafish model for evaluating effects of NETA and EE in human pregnancy;
- the robustness of the study; and
- any clinical implications.

3. Background

3.1 Products containing NET(A) and EE

Hormone Pregnancy Tests (HPTs) were widely used to diagnose pregnancy from the late 1950s until 1978. The most frequently used HPT in the UK, Primodos, contained two hormones – norethisterone acetate (NETA;10mg per tablet) and ethinylestradiol (EE; 0.02mg per tablet). NETA, a prodrug of norethisterone (NET), is a progestogen derived from nortestosterone that also has weak oestrogenic and androgenic properties. EE is a synthetic estrogen with actions similar to those of natural estradiol. One Primodos tablet was taken on two consecutive days by women suspected to be pregnant. In women who were not pregnant, a withdrawal bleed would occur a few days later. A conservative estimate of the likely window for use of Primodos was from the week of the woman's first missed period to the end of the first trimester; that is, 4 to 12 weeks of pregnancy (2 to 10 developmental weeks). Other HPTs similarly contained high doses of a progestogen and an estrogen.

Although Primodos has not been marketed in the UK for 40 years, NETA or NET are currently the progestogenic component of a number of oral contraceptives and hormone replacement therapies, and a common treatment for menstruation disorders (**Table 1**). Typical daily doses range from 0.35mg per day in Noriday contraceptive to 60mg per day in Utovlan, a treatment for disseminated breast cancer. Similarly, EE is frequently used as the oestrogenic component of combined oral contraceptive preparations; a typical daily dose is 20 to 40 µg.

No currently licensed medicines in the UK contain the same combination and dosage of progestogens and estrogens as were present in HPTs such as Primodos. However, varying

[1] Brown S, Fraga LR, Cameron G, Erskine L, Vargesson N. The Primodos components Norethisterone acetate and Ethinyl estradiol induce developmental abnormalities in zebrafish embryos. Sci Rep. 2018; 8: 2917. https://www.ncbi.nlm.nih.gov/pubmed/936986

combinations and dosages of similar progestogens and estrogens are used daily by many millions of women.

Table 1 Examples of oral medicines containing NET(A) in combination with EE or estradiol

Product name	Progestogen/estrogen (per tablet)	Posology	Indication
Loestrin 30	NETA 1.5 mg EE 30 µg	1 tablet daily for 21 days per cycle	combined oral contraceptive
Norimin	NET 1mg micrograms EE 35 µg	1 tablet daily for 21 days per cycle	combined oral contraceptive
Noriday 350	NET 350 µg	1 tablet daily	progestogen-only oral contraceptive
Primolut N	NET 5 mg	10-15 mg daily for 4-6 months	endometriosis
		15 mg daily for 10 days	dysfunction uterine bleeding menorrhagia
		15 mg daily for 3 days	postponement of menstruation
		15 mg daily for 20 days	dysmenorrhoea
Utovlan	NET 5 mg	40 - 60 mg daily	disseminated carcinoma of the breast
Elleste Duet Conti	NET 1mg estradiol 2mg	1 tablet daily	hormone replacement therapy (HRT)
Primodos	NETA 10 mg EE 20 µg	1 tablet on each of 2 consecutive days	Hormone pregnancy test - discontinued

3.2 Regulatory approach to assessment of the developmental toxicity of drug substances

Non-clinical studies are central to the prediction of human toxicity. The non-clinical data that are required for the assessment of potential developmental toxicity of a new medicine is described in International Conference for Harmonization (ICH) Technical Requirements for Pharmaceuticals for Human Use M3 ‘Guidance on Non-Clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals’ and more specifically in ICH S5 (R3) ‘Revision of S5 Guideline on Detection of Toxicity to Reproduction for Medicinal Products & Toxicity to Male fertility’. The current ICH5 S5 (R2) guideline only considers mammalian species for the assessment of reproductive studies. The rat and rabbit are the routine primary and secondary species for the determination of developmental toxicity. Non-human primates can be used for evaluating developmental

toxicity if they are the only pharmacologically relevant species. The revised ICH S5 (R3), which in draft form, considers that validated alternative non-mammalian assays can be used where they are interpreted in conjunction with existing in vivo mammalian models. It goes on to state that for appropriate use of alternative assays it is important to establish the reliability and predictivity for in vivo reproductive outcomes and sets out guidelines for qualification of alternative assays for regulatory use.

In considering the robustness of the evidence of a potential signal for developmental toxicity of a marketed medicine raised from a non-regulatory non-clinical study, there are several considerations that have to be taken into account. It is important to consider the physiological and pharmacological relevance of the animal model(s) used. Also, to critically consider the extent and timings of the exposures obtained in the non-clinical study and the translational relevance of the findings to fetal exposure via the maternal route during human pregnancy. All these factors are explored in this paper.

Regulatory reproductive toxicity studies are conducted according to good laboratory practice (GLP) standards which ensure the quality of the data. ICH S5 (R3) considers that alternative assays should generally be conducted to GLP when part of a risk assessment. If a study is exploratory in nature a judgment must be made regarding the quality, conduct and reporting of the study. For example, studies involving animals should clearly present key aspects of the experimental design such as randomisation of animals, whether blinded assessment of outcomes was conducted, sample size calculations and the statistical methods used.

One of the most critical considerations when reviewing the translational relevance of non-clinical studies is the consideration of the levels of drug exposure in animals. Species differences in response to a drug often relate to differences in the pharmacokinetics that determine the concentration/time relationship of exposure to the relevant tissue/organ. The pharmacokinetics of a substance can be affected by a range of factors including how much it is bound to proteins in the circulation, how it is distributed within the body, how it is metabolised and whether it crosses membrane barriers and if so, to what extent. For example, knowing whether toxicities are due primarily to the parent drug or metabolite is important in the risk assessment. The comparison of systemic exposure and drug metabolism between test species and humans is an important element to consider when assessing the potential risk of an identified hazard occurring in humans.

If an adverse effect is recognised, a key aspect of evaluating the potential risk to humans is determining the systemic exposure (C_{max}/AUC) and duration of exposure associated with toxicity and to determine if it is induced at or near clinically relevant exposures. A ‘safety margin’ can be derived by comparing the maximum exposures at which observed adverse effects did not occur in the nonclinical study (No Observed Adverse Effect Level: NOAEL) with the systemic exposure in humans at the maximum recommended dose.

3.3 Utility of zebrafish models in developmental toxicity testing

Over the past two decades the use of zebrafish in biomedical research has been increasing. Zebrafish (*Danio rerio*) models have some advantages as an alternative assay for developmental toxicity testing and can serve to complement the standard regulatory mammalian assays recommended in ICH S5 (R3). Zebrafish assays have replicated the effects of some known developmental toxicants such as thalidomide. Other attributes such as very well characterised embryonic and laval development and rapid development with organogenesis complete in 3 days also make them a useful alternative. The model has demonstrated some utility in hazard identification although the lack of maternal influences,

placental function and pharmacokinetics may limit the extrapolation of reproductive findings to humans (Brannen et al 2010). The model is used predominately to evaluate direct effects on isolated embryos, although some studies have investigated maternal passage of effects to offspring (Lai et al., 2017). In pharmaceutical development to date they have been used mainly for medium- to high-throughput toxicity screens. Zebrafish are used extensively as a model fish species for evaluating effects of environmental pollution.

4. Research for consideration

"The Primodos components Norethisterone acetate and Ethinyl estradiol induce developmental abnormalities in zebrafish embryos.' (Brown et al. 2018)"

The study by Brown et al.[2] is described and, where relevant, reference is made to relevant existing non-clinical, pharmacology and pharmacokinetic data available in the public domain.

Brown et al. performed a series of exploratory experiments to observe the effects of a mixture of NETA and EE on embryo development and survival for which dechorionated zebrafish embryos were exposed. In such experiments, embryos were incubated with NETA/EE at varying concentrations, at different time points for varying durations to determine effects. Additional experiments were performed to validate such findings, which included determining the accumulation of NETA in zebrafish embryos, effects on blood vessel patterning, and effects on nerve patterning and outgrowth following exposure to NETA/EE.

Seven experiments are described in the paper. The methodology, findings and conclusions of the authors for each experiment have been summarised, and points that the Expert Group may wish to consider have been highlighted.

i. "A NETA and EE mixture impairs zebrafish development and survival in a dose responsive manner"

The authors performed an experiment to observe effects on the development and survival of dechorionated zebrafish embryos following exposure to a range of NETA/EE-mixture concentrations.

Method:
Embryos (n≥15/group) were hand dechorionated and exposed to a NETA:EE mixture at 24 hours post-fertilisation (hpf) and incubated for 72 hrs. The NETA:EE mixture was prepared at a 500:1 ratio, a ratio equivalent to that of Primodos, at six concentrations increasing incrementally from 1.5 µg/mL NETA + 3.125 ng/mL EE to 50 µg/mL NETA + 100 ng/mL EE. Dimethyl sulfoxide (DMSO) was added to solubilise NETA/EE mixture yielding a final DMSO assay concentration of 0.2%.

A 24 hpf time point was chosen as this developmental time point is the period where most tissues and organs are rapidly developing and has been used in previous work in the laboratory analysing drug actions upon embryogenesis. Moreover, the authors state this time period relates to approximately weeks 6–10 in human embryo development when hormonal pregnancy tests were likely to be used.

[2] https://www.ncbi.nlm.nih.gov/pubmed/29440757

Results:
No effect on embryonic development and survival was observed at the two lowest doses tested (1.5 µg/mL NETA + 3.125 ng/mL EE and 3.125 µg/mL NETA + 6.25 ng/mL EE).

At 6.25 µg/mL NETA + 12.5 ng/mL EE (n = 62), gross embryonic defects (approx. 20% of embryos) and a small increase in embryonic death (approx. 10% of embryos) was observed. Specifically, length of the pectoral fins and overall body length were decreased significantly.

At 12.5 µg/mL NETA + 25 ng/mL EE (n = 91) markedly more embryos displayed damage (approx. 92% of all embryos had damage) and almost 50% of the embryos died. Specifically, length of the pectoral fins, overall body length, eye size and otic vessel size were decreased significantly (15-17%). Additional malformations including bent spine, smaller overall size, pericardial and yolk sac oedema and oedematous yolk sac extension were also observed at this concentration.

Doses of 25 µg/mL NA + 50 ng/mL EE or higher caused 100% lethality (n = 23).

Author's conclusions:
This data demonstrates that the NETA/EE-mixture impairs embryonic development and induces embryo lethality in a dose dependent manner.

Points for consideration:

Duration of exposure
In the first experiment, zebrafish embryos were exposed to NETA/EE at 24 hours post fertilization (hpf) and incubated with NETA/EE solutions for up to 72 hours since this time period is said by Brown et al. to correlate to approximately 6-10 weeks in terms of human development. A 72 hour period of incubation with NETA/EE would equate to a longer duration of exposure than the two consecutive daily doses of Primodos that were taken by pregnant women around weeks 6-10 of pregnancy.

State of embryo
The zebrafish embryo is surrounded by the chorion, an acellular envelope. It would be valuable to understand what effect manual dechorionation to expose the 'naked' embryo may have had on exposure to NETA/EE.

Pharmacokinetics and route of exposure
How direct constant exposure of dechorionated zebrafish embryos to high drug concentrations in a 'closed system' compares to the maternal-fetal pharmacokinetic profiles of NETA/EE described following oral dosing in mammalian systems (see Discussion).

Choice of vehicle
DMSO was used to solubilise NETA/EE at a final concentration of 0.2% v/v. This is 20-fold higher than the maximum concentration of 0.01% recommended in the OECD fish embryo acute toxicity test guidance document (OECD Test #236). A study by Xiong et al. suggests the no-observed-effect-concentration (NOECs) for gross developmental effects in zebrafish embryos for DMSO is 1.0% v/v and the NOECs for induction of stress proteins is 0.001% (Xiong et al., 2017). DMSO is a known penetrant enhancer (Kais et al., 2013), which could further increase exposure of NETA/EE to the embryo.

Availability of 'free' NETA/EE
In human plasma at normal circulating levels NET and EE are predominately bound (>97%) to serum albumin and/or the sex hormone binding globulin (SHBG) which regulate the amount of biologically active NET/EE available. It is not clear what effect an absence of

protein in the zebrafish medium might have on the concentrations at which the observed developmental effects are observed.

Concentration effect threshold
The minimum concentration at which effects on development and survival were observed was 6.25 µg/mL NETA + 0.0125µg/mL EE. This equates to a conservative exposure margin of 84-fold based on the maximum total drug concentration in human plasma following 2 doses of Primodos (or 3,125-fold based on the drug free fraction). See Discussion for further detail.

Adult zebrafish studies
Chandrasekar et al., (2010) showed 100% lethality of estradiol following in vivo exposure of adult zebrafish for 48 h at 5 µM (1.8µg/ml). It would be valuable to confirm if any data on the possible toxicity of NETA/EE in juvenile/adult zebrafish has been obtained.

Reversibility
It would be valuable to understand if any of the effects observed in this study are reversible upon removal of the NETA/EE solution.

ii. "Embryos at earlier developmental stages are more sensitive to the NETA/EE-mixture"

The authors performed an experiment to observe the effect of a single predefined concentration of NETA/EE-mixture on the development of dechorionated zebrafish embryos at different developmental stages.

Method:
In this experiment, hand-dechorionated embryos were exposed to 12.5 µg/mL NETA + 0.025 µg/mL EE at 6, 24, 48, and 72 hpf for 24 hours. Dose selection was based on the effects that were observed in the previous dose-response study i.e. 57% survival rate with the majority (92%) of embryos presenting defects at this dose.

Results:
Embryos exposed at 6 hpf exhibited severely malformed tails and bent spines, malformed pericardial sacs, yolk sac damage/oedema and very small eyes, whereas embryos exposed at 48 hpf and at 72 hpf, had less severely bent spines, mild pericardial defects and their eyes and otic vesicle appeared to be normal; the only consistently observable effect was an oedematous yolk sac.

Author's Conclusions:
This indicates that early stage embryos are more sensitive to the NETA/EE-mixture than later stage embryos.

iii. "Exposure to the NETA/EE-mixture causes rapid morphological damage"

The authors performed an experiment to determine how quickly a single predefined concentration of NETA/EE-mixture induced embryonic damage in dechorionated zebrafish embryos.

Method:
Hand-dechorionated embryos were exposed to 12.5 µg/mL NETA + 25 ng/mL EE at 24 hpf for different time points from 1 to 24 hours.

Results:
The first distinct morphological damage (reduced eye size and body length) was evident from 4 hrs after NETA/EE-mixture application. The forming heart also showed changes by 4 hrs of incubation in a subset of embryos (n = 2/5). Other tissues, such as the yolk sac, appeared unaffected at this timepoint.

Inhibition of embryonic movement (tail movement, embryo start vs. final position) was also observed at all timepoints assessed up to and including 4 hrs of exposure (n ≥ 5).

Author's Conclusions:
This indicates that the NETA/ EE-mixture acts rapidly upon the embryo, with some tissues more susceptible than others, and demonstrates that short-term exposure to the NETA/EE-mixture in zebrafish induces significant defects in embryonic development and movement.

Points for consideration:

Relationship to humans
Zebrafish hatch at 3-4 days after fertilisation. The 4 hours exposure to NETA/EE in this study is therefore estimated to equate to approximately one week or more of human embryo development.

Movement effect
After 1 hr of exposure there was a drastic decrease in movement in NETA/EE-mixture treated embryos. The rapid onset of this effect may suggest interactions with receptors/ion channels other than nuclear steroid receptors and possibly interactions with targets controlling neuronal activity.

iv. "Quantification of the dose of the drug that reaches the embryo"

The authors performed an experiment to determine the amount of NETA in zebrafish embryos at numerous timepoints following exposure to a single predefined concentration of NETA/EE-mixture.

Methods:
NETA was dissolved in water at a concentration of 1 mg/mL and stored in aliquots at −20 °C. Quality control samples were prepared in water/methanol (50/50) at 10, 85 and 175 ng/mL NETA and stored at −70 °C. Daily, NETA was diluted in water/methanol (50/50) to give calibration standards in the range 6.25–200 ng/mL.

For this analysis, levels of NETA were assessed because EE levels were consistently below detection rates. Embryos at 24 hpf were placed in the NETA/EE-mixture (12.5 µg/mL NETA + 25 ng/mL EE), or in DMSO or in water (untreated) for 6 hr, 24 hr or 48 hr. Embryos were rinsed in water and then frozen before Mass Spectroscopy analysis.

Results:
The level of NETA in the embryos was 1 µg/embryo with 6 hr of dosing, peaking at 1.8 µg/embryo with 24 hr of dosing and subsiding to 1.2 µg/embryo with 48 hr dosing (Fig. 4).

Author's Conclusions:
This data indicates that NETA can accumulate in embryonic tissue for at least 24 hrs.

Points for consideration:

Distribution and uptake in embryo
Under the assay conditions used, high levels of NETA are associated with the zebrafish embryo (1 - 1.8 µg per embryo), although the distribution throughout the embryo has not been quantitated. The apparent high levels of uptake may be related to the highly lipophilic nature of NETA. NETA and EE have logP values of 3.5 and 3.9 respectively and very low aqueous solubility (6µg/ml and 11µg/mL respectively at 25^{o}C). De Koning et al., (2015) showed that exposure to compounds with higher logP values results in higher uptake into the zebrafish embryo. De Koning et al., (2015) showed that for lipophilic compounds, >90% of the compound is taken up by the embryo.

The average wet weight of the zebrafish embryos evaluated at 24 to 72 hpf is needed to be able make a more precise estimate of the potential for accumulation of NETA. The average wet weight of a 144 hpf zebrafish embryo is just less than 2mg (Avella et al., 2012). The results by Brown et al. show that 1 µg and 1.8 µg of NETA per zebrafish embryo was measured at 6 and 24 hours, respectively (corresponding to 30 and 48 hpf). Therefore, the amount of NETA per zebrafish embryo at the two different time points is conservatively estimated as 500 to 900 µg/g wet weight tissue, based on a 2mg wet weight embryo. This equates to an accumulation ratio of ≥40-fold (12.5 µg/ml to ≥500 µg NETA/g wet weight).

Maximum drug exposure threshold
Evaluations of the potential of zebrafish to be a useful screen for classification of teratogenicity and/or embryotoxicity have suggested a maximum drug exposure threshold of 30 ng/embryo when classifying teratogenic compounds to avoid 'overdose' levels (van den Bulck et al., 2011). In the study by Brown et al. the amount of NETA per zebrafish embryo is 1-1.8 µg/embryo between 6 to 48hrs of incubation.

Drug delivery via embryo medium
In an orally dosed mammalian maternal-embryofetal drug distribution system the drug is cleared from both the maternal and fetal compartments. The zebrafish uptake assay is a 'closed' system with a constant high drug concentration in the external embryo media compartment.

Linearity of uptake
A range of exposures would need to be evaluated to obtain additional information on the linearity or otherwise of the uptake of NETA. Given the high concentrations of NETA/EE evaluated, it is possible that potential efflux mechanisms, protein binding sites and the ability to metabolise drug are saturated.

v. NETA/EE-mixture exposure increases cell death and reduces cell proliferation throughout the embryo

The authors performed experiments to investigate if a single predefined concentration of NETA/EE-mixture affected cell death and proliferation in dechorionated zebrafish embryos to potentially explain the damage and phenotypes observed.

Method:
Embryos were treated with NETA/EE-mixture (12.5 µg/mL NETA + 25 ng/mL EE), or DMSO at 24 hpf and fixed at 6 hrs and 24 hrs after exposure (n ≥ 5/condition and time-point). Cell death was performed using a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay whereas cell proliferation was assessed by staining for Phospho-histone H3, a marker of mitosis.

Results:
At both time points cell death was increased significantly in embryos treated with the NETA/EE-mixture ($p < 0.01$). Cell death was not localised to specific tissues but was observed throughout the embryo.

A decrease in the number of mitotic cells at both 6 hr and 24 hr in embryos exposed to the NETA/EE-mixture compared to controls was also observed. Similar to the cell death analyses, no regional variations in cell proliferation were observed, but there was a general decrease in cell proliferation throughout the embryo in NETA/EE-treated embryos.

Author's Conclusions:
NETA/EE promotes non-specific cell death and a reduction in cell proliferation in the zebrafish embryo.

Points for consideration:

The possible reasons for the observed non-localised increases in cell death and reduced cell proliferation at a concentration of 12.5 µg/mL NETA + 0.025 µg/mL EE.

vi. NETA/EE-mixture exposure alters embryonic blood vessel patterning

The authors performed experiments to investigate if a predefined concentration of NETA/EE-mixture affected blood vessel patterning in dechorionated zebrafish embryos. A subsequent in vitro experiment was also performed in human endothelial vein umbilical cells (HUVEC) using predefined concentrations of NETA/EE-mixture. The purpose of these experiments was to investigate if a NETA/EE-mixture affected blood vessel patterning to potentially explain the embryo-developmental effects observed.

Method:
Blood vessel loss or patterning defects in zebrafish embryos following NETA/EE exposure was investigated used the transgenic fli1:EGFP reporter line of zebrafish embryos. Embryos were exposed to either DMSO or 12.5 µg/mL NETA + 25 ng/mL EE at 24 hpf and their intersomitic vessels (ISV) imaged at 6 and 24 hours after exposure.

Because intersomitic vessel defects could be secondary, for example due to changes in somite formation which have been shown to be the cause of vessel positioning changes in Notch signalling pathway mutants, the effect of NETA/EE-mixture exposure directly using in vitro cultures of cells from a human umbilical vein endothelial cell line (HUVEC) was next studied. Briefly, HUVEC cells were exposed to three concentrations of NETA/EE mixture (6.25 µg/mL NETA + 12.5 ng/mL EE, 12.5 µg/mL NETA + 25 ng/mL EE) or 0.02% DMSO as a control. The cells were incubated for 18 hr at 37 °C, fixed in 4% PFA for 1 hour and stained with antibodies for Phosphohistone H3.

Results:
Embryos incubated with DMSO displayed complete dorsal vessel anastomosis 24 hours after exposure (48 hpf), comparable to untreated wild-type embryos.

In contrast, incubation with the NETA/EE-mixture caused some mispatterning of vessels within 6 hr of exposure and misplacing, mispatterning and stunting of intersomitic vessel outgrowth throughout the spine of the embryo at 24 hr following drug exposure.

Quantification of intersomitic vessel outgrowth demonstrated no outgrowth deficit at 6 hr but significant reduction in outgrowth by 24 hr.

Application of the NETA/EE-mixture to newly plated HUVEC cells before the HUVEC cells have formed endothelial cell tubes, caused changes to the number of branches of endothelial tubes in a dose-sensitive manner. Cell proliferation and cell number also was decreased in a concentration dependent manner (significant at 12.5 µg/mL NETA + 25 ng/mL EE). Despite the number of endothelial cells and their proliferation rates being reduced at each concentration, they were still able to form patterned, branched, vascular networks, though bigger gaps are seen between the endothelial tubes.

Author's Conclusions:
This data indicates that vessels can form in the presence of the NETA/EE-mixture but endothelial cell proliferation and vessel branching is perturbed.

Points for consideration:

Effects on HUVECs and the ability to form vessel like structures in vitro occurred at similar concentrations to effects on growth and proliferation of HUVECs. Thus, effects on vessel like formation may represent a secondary effect related to reduced proliferation/viability of cells at this dose.

Inhibition of proliferation of human endothelial cells in culture has been previously demonstrated with progesterone at 1 µM concentrations (Vázquez et al., 2007). Studies examining pharmacological effects of NETA in mammalian cell culture are generally conducted in the nM range (Brosnan et al., 2013).

vii. NETA/EE-mixture exposure affects nerve patterning and outgrowth in vivo and in vitro

The authors performed experiments to investigate if a predefined concentration of NETA/EE-mixture affected nerve patterning and outgrowth in dechorionated zebrafish embryos. A subsequent in vitro experiment was also performed using mouse retinal explants using predefined concentrations of NETA/EE-mixture to confirm whether the neuroinhibitory action observed in the zebrafish embryos was direct. The purpose of these experiments was to investigate if a NETA/EE-mixture affected the nervous system to potentially explain the phenotypes of bent spine and reduced movement in embryos exposed to NETA/EE.

Method:
Embryos were treated with either DMSO or NETA/EE (12.5 µg/mL NETA + 25 ng/ mL EE) at 24 hpf, fixed at 6 hr and 24 hr and then stained with an anti-neurofilament antibody to analyse nerve patterning.

To confirm whether any neuroinhibitory action observed in the embryos was direct, the effects of the NETA/EE-mixture exposure on neurite outgrowth was investigated using an *in vitro* mouse retinal explant assay. Retinas were dissected from E14.5 C57BL/6 J WT mice and cultured in DMSO or in a range of concentrations of NETA/EE (3.125 µg/mL NETA + 6.25 ng/mL EE; 6.25 µg/mL NETA + 12.5 ng/mL EE; and 12.5 µg/ mL NETA + 25 ng/mL EE). After 48 hr, the cultures were fixed and stained with a neuron-specific anti–β-tubulin antibody and the area of neurite outgrowth from the cultures quantified.

Results:

Embryos treated with NETA/EE (n = 9 for 6 hr and n = 16 for 24 hr) presented defasciculation of axons in the developing spinal cord and shortening of axonal outgrowth. Axons had not extended to the midpoint of the spine by 6 hrs and failed to innervate the tail region of the embryo by 24 hr. Quantification of nerve length relative to overall body length indicated significant nerve length reduction from 6 hrs following drug exposure. By contrast, in DMSO treated control embryos axons could be seen extending through the spinal cord to midway through the spine after 6 hrs and throughout the spine by 24 hr exposure. In the developing head of the embryo nerves were also disorganised, mispatterned and defasiculated when compared to embryos treated with DMSO (n = 7 for 6 hr and n = 12 for 24 hr). Total nerve outgrowth in the head also was reduced significantly in treated embryos.

Results from the retinal explant assay revealed that NETA/EE had a dose-dependent inhibitory effect on neurite outgrowth. Treatment with 3.125 µg/ mL NETA + 6.25 ng/mL EE had no significant effect on neurite outgrowth (n = 14) when compared to DMSO (vehicle) controls (n = 15). However, the extent of neurite outgrowth from retinal explants exposed to 6.25 µg/mL NETA + 12.5 ng/mL EE (n = 13) and 12.5 µg/mL NETA + 25 ng/mL EE (n = 13) was decreased significantly compared to DMSO controls.

Author's Conclusions:
These findings demonstrate that the NETA/ EE-mixture can inhibit nerve outgrowth when directly applied to in vitro nerve explants and can also cause axonal outgrowth defects in vivo.

Author's discussion:

A paraphrased version of the Brown et al. conclusions is presented in the paragraphs below.

These studies demonstrate that a mixture of NETA/EE (the components of Primodos), can cause developmental anomalies when directly applied to zebrafish embryos. The compound acts in both a dose-dependent and time sensitive manner, with early exposure causing more damage than later exposure. Damage also is extremely rapid. Within 1 hr of drug exposure at 24 hpf, embryos displayed significantly reduced movement, and, within 4 hrs of exposure, obvious morphological defects. Using *in vitro* assays utilising human HUVEC cells and mouse retinal explants NETA/EE exposure was observed to directly impair blood vessel pattern formation and nerve outgrowth. These findings demonstrate that the components of Primodos are potentially teratogenic, affecting the development of a wide range of zebrafish organ systems *in vivo* and further provide evidence that these components can affect the development of mammalian tissues in vitro.

The concentrations chosen to investigate the action of the NETA/EE-mixture upon embryonic development (12.5 µg/mL NETA + 25 ng/mL EE) was determined from carrying out a dose response analysis. At the 24 hpf developmental timepoint lower doses had no effect, whilst higher doses caused severe damage or death. The peak of NETA in human plasma averages 18.3 ng/mL, or 0.0183 µg/mL, when a 1 mg dose is taken and averages 26 ng/ml in human plasma within 1–2 hours of administration of a 5 mg dose. Considering that when used as a pregnancy test, the dose of Primodos taken was 10 mg, it is expected that the circulating NETA would be higher. Moreover, given the normally elevated levels of progesterone and oestrogen during pregnancy, (from 10–54 ng/mL and 486–1615 pg/mL respectively) the use of a synthetic progesterone-based hormone will result in a total higher concentration in pregnant versus non-pregnant women.

Mass spectroscopy was used to measure the levels of NETA within the zebrafish embryos and found that in just 6 hrs the concentration was 1 µg/embryo and the concentration peaked at 1.8 µg/embryo after 24 hr incubation. The doses used are therefore higher than

the plasmatic dose seen in humans after Primodos exposure. However, the receptor specificity or transport ability of these synthetic human hormones in zebrafish is not known and could be significantly different. Thus, the authors state that it is difficult to extrapolate from this work what would be a teratogenic dose in humans.

Moreover, in human plasma the half-life of NETA, a synthetic progestogen, is much longer (up to 9 hours) than endogenous progesterone (reportedly 5 mins). Even though the drug would then dilute throughout the blood plasma and likely be metabolised in the maternal liver, a study investigating NETA/EE uptake in early human pregnancies showed levels of NETA in the maternal blood plasma were elevated for up to 48 hrs after exposure, however, levels of NETA/EE in the embryos were not described. The authors hypothesize that because there is little to no metabolic liver function in early embryogenesis it is possible that the drug concentration will accumulate and build up to high levels in the human embryo over time, as has been observed in the zebrafish embryos.

Progesterone also is known to be metabolised into corticosteroids such as cortisol by the foetal kidney and adrenal gland, which if found at high levels has teratogenic effects in zebrafish, and mammals such as sheep. This raises the possibility that exogenous progesterone, and synthetic forms like NETA, might metabolise into these teratogenic compounds, and thus cause developmental defects within the embryos.

Several known teratogens including thalidomide and valproate as well as anticancer antiangiogenic drugs are thought to cause embryonic damage through vessel inhibition. However precisely how loss of vessels results in embryonic damage remains unclear and further work is needed. Recent work has shown that progesterone and oestrogen regulate expression of vascular regulators including VEGF and angiopoietin in human and in primate endometrium. Taken altogether, this suggests that NETA/EE can induce embryological defects through a range of mechanisms including, impaired nerve growth and angiogenesis, elevated cell death and impaired cell proliferation.

It is difficult and dangerous to directly compare drug action/s between species. Nevertheless, these data demonstrate accumulation of the drug in the embryo, which does not decrease for some time, and leads to rapid embryonic damage. From other animal models of drug-induced teratogenesis, for example thalidomide exposure, higher doses, than used in humans, are required to reciprocate the damage seen in humans due to differences in applications, uptake and metabolism.

Whether the zebrafish progesterone and oestrogen receptors bind synthetic human progestogen (NETA) and oestrogen (EE) compounds with a similar affinity to human progesterone and oestrogen receptors is unclear, and this might contribute to why high doses of the NETA/EE-mixture were needed to see zebrafish embryonic damage. The authors conclude that more work is required in mammalian species to confirm these findings.

5. Assessor's discussion of the data

This set of exploratory studies by Brown et al. demonstrate that under certain conditions a combined mixture of NETA and EE added to the incubation medium of dechorionated zebrafish embryos induces certain developmental defects and that the extent of such developmental effects is dose- and time-dependent. The authors have further investigated the potential underlying mechanisms involved in the observed developmental effects by revealing NETA/EE-induced effects on blood vessel pattern formation and nerve outgrowth in subsequent experiments using *in vivo* and *in vitro* models.

In understanding the potential relevance of these findings to the development of human embryos a number of aspects need to be considered as follows.

5.1 Translational relevance of the zebrafish model for evaluating effects of sex hormones in humans

In considering the relevance of the model used by Brown et al. to human reproduction, it is acknowledged that the zebrafish model has some advantages as an alternative to mammalian models for developmental toxicity testing and can serve as a complement to standard regulatory mammalian assays. Thus:

- zebrafish assays in which embryos have been directly exposed to known developmental toxicants such as thalidomide have replicated the effects observed in mammalian assays
- zebrafish embryonic and larval development are well characterised and rapid, with organogenesis complete in 3 days. This makes them ideal for medium- to high-throughput toxicity screens in pharmaceutical drug development.

Compared with mammalian models used for human developmental toxicity risk assessment the zebrafish model lacks maternal influences, placental function and pharmacokinetics which has been said to limit the extrapolation of reproductive findings to humans (Brannen et al., 2010). The Expert Group may wish to consider the issues associated with translating the effects of sex steroids such as progestogens in zebrafish to humans when the major effects in human reproduction relate to effects on the maternal endometrium, embryo implantation and maintenance of pregnancy.

5.2 Fish progestogen receptor pharmacology

The similarity in the pharmacology of the model system to humans is a key consideration when choosing an appropriate toxicological assay. The current draft ICH guidance on reproductive toxicity studies (ICHS5(R3)), states: “Additional points to consider in selection of a species relate to the interaction of the pharmaceutical with the species include:

a. The pharmacokinetic and metabolite profile (including adequate exposure to major human metabolites, as discussed in ICH M3(R2) (1));
b. Whether the species expresses the pharmacologic target (e.g., is an endogenous or exogenous target) and whether the pharmaceutical has adequate affinity for the target in the species selected;
c. Whether the functional pharmacological activity of the pharmaceutical is exhibited in the test species.”

In fish, progestogens play an important role in the stimulation of oocyte final maturation and ovulation in females, stimulation of spermiation and sperm motility in males, and the initiation of meiosis in both sexes. However, studies have suggested that receptor pharmacology of the progestogens in fish differs from their human counterparts.

Progestogenic effects:

In humans progesterone (P4) is the major progestogen; in fish 17,20β,-dihydroxy-4-pregnen-3-one (DHP) and 17,20β,21-trihydroxy-4-pregnen-3-one are the endogenous teleost progestogens (Ellestad et al., 2014).

19-nortestosterone-derived progestogens such as NETA as well as having potent progestogenic activity, also have androgenic effects in mammalian systems. In vitro reporter gene transactivation assays driven by the fathead minnow androgen receptor have revealed potent activation by 19-nortestosterone-derived progestogens including NET (Ellestad et al., 2014; **Table 2**). However, in contrast to other synthetic progestogens none of the 19-nortestosterone derived progestogens tested, including NET, activated the fathead minnow progestogen receptor. Consistent with this finding Bain et al., (2015) showed that all 19-nortestosterone derivatives including NET were potent agonists of the human progestogen receptor but did not activate the rainbow fish progestogen receptor. In contrast all 19-nortestosterone derivatives including NET were potent agonists of the fish androgen receptor with EC_{50} in the range 2-10nM. This suggests that unlike the human nuclear progesterone receptor, which is more potently activated by most synthetic progestogens than by natural progesterone, 19-nortestosterone-derived progestogens do not act as agonists of the fish nuclear progestogen receptor.

Table 2. Potency of selected progestogens determined for the human, fathead minnow or rainbowfish progesterone nuclear receptor or androgen receptor (Ellestad et al., 2014 and Bain et al., 2015)

Ligand	Progesterone receptors (EC_{50} in nM)			Androgen receptors (EC_{50} in nM)		
	human	rainbow fish	fathead minnow	human	rainbow fish[a]	fathead minnow
DHP (fish progestogen)	NMA	8.27	0.74	NMA	NMA	NMA
norethisterone	1.55	NMA	NMA	8.1	0.83	0.12
progesterone	5.81	132	36.6	NMA	NMA	25.5

[a] Data obtained from alpha receptor subtype

Abbreviations: DHP = 17,20β,-dihydroxy- 4- pregnen-3-one; EC_{50} = half maximal effective concentration; NMA = no measurable activity

Signalling through the androgen receptor is consistent with studies of the pharmacodynamic actions of NET in fish, which have shown that exposure to NET at or above 25pg/mL causes a significant reduction in fecundity in medaka breeding pairs and induces male colouration in female fathead minnow (Paulos et al., 2010). In developing zebrafish embryos from 48hpf, changes in the expression of selected nuclear hormone receptors occur in response to levels of NET as low as 2pg/ml (Zucchi et al., 2012).

In referring to reproductive effects observed with low hormonal concentrations in earlier studies, Brown et al. suggest that "*the difference in concentrations required to induce embryonic damage in this study compared to previous work likely reflects the shorter time course of drug application in our study. In keeping with this idea, we have shown that the drug accumulates in embryos over time. Thus, prolonged exposure will likely result in higher concentrations within the embryo as development proceeds*." For some reproduction endpoints such as effects on fecundity longer exposures times have been used. Other studies with zebrafish embryos have followed exposure times equivalent to those used by Brown et al. and shown pharmacodynamic effects in the pg/ml range (Zucchi et al., 2012).

The published concentration range for the primary pharmacodynamic actions of synthetic progestogens in fish is in the pg/ml range (Zeilinger et al., 2009; Paulos et al., 2010; Runnalls et al., 2013; Svensson et al., 2013; Han et al., 2014; Liang et al., 2015). The data presented by Brown at al. on NETA accumulation investigated concentrations of NETA up to 12.5 µg/ml which resulted in high levels in embryos (1 – 1.8 µg /embryo equivalent to ≥ 500µg /g tissue) between 6 to 48hrs.

Estrogenic effects:

It is known that estradiol binds the zebrafish estrogen receptor (ER) with calculated K_d close to the ER K_d in mammals (0.2-0.4 nM) (Menuet et al., 2002). There is an established literature available on effects of low pg/ml levels of ethinylestradiol on zebrafish as it is used as a model endocrine disrupting molecule in fish populations. These data suggest that the translational relevance to humans of effects observed with low pg/ml doses of EE in zebrafish are more straightforward than with progestogens; there are few published data on the effects of EE in higher (ng/ml) concentrations.

Summary

Detailed pharmacology studies in zebrafish and related species suggest that 19-nortestosterone-derived progestogens such as NETA are likely to elicit strong androgenic activity in bony fish such as zebrafish and minimal nuclear progesterone receptor-mediated effects. The Expert Group may want to consider the challenges in translating NETA-induced developmental effects via progesterone-receptor mediated pathways from zebrafish to humans.

5.3 Exposure concentration of drug in medium

The published pharmacodynamic effects of NETA and EE in both embryo and adult fish at pg/ml concentrations are consistent with the known pharmacology of NETA/EE and in certain ways reflect effects seen in humans i.e. effects on fertility and masculinization of female fetuses following extended exposure with NET.

The developmental effects seen by Brown et al. start at concentrations of NETA and EE in the external embryo media of 6.25 µg/mL NETA + 0.0125 µg/mL EE (NETA 18.4µmol/L + EE 0.04µmol/L). At these concentrations a >40-fold accumulation ratio in the embryo tissue has been demonstrated. Given the known primary pharmacology of NETA the question is whether the rapid effect on movement (within 1 hour of exposure) and the effects on multiple aspects of development in different tissues are mediated through nuclear sex steroid receptors or other receptors.

Both NETA and EE are highly lipophilic compounds with low aqueous solubility with logP (a partition-coefficient that measures lipophilicity) values of 3.5 and 3.9 respectively. In human plasma at normal circulating levels NET is predominately bound (>97%) to serum albumin and the sex hormone binding globulin (SHBG) which regulate the amount of biologically active NET available. The solubility of NETA is around 6µg/ml at 25^{o}C in pure water (Ran et al., 2002) - approximately the same as the concentrations at which developmental effects were seen in the zebrafish studies. Given NETA's biophysical properties and the high concentrations used, which are not found following the dosing of humans, NETA may be expected to bind with low affinity (non-specifically) to hydrophobic surfaces and could saturate both specific and non-specific binding sites. The Expert Group may wish to consider if these interactions could lead to non-specific pharmacological effects.

NETA is rapidly metabolised to its active component, NET, in mammals including humans; it is not clear to what extent this happens in this zebrafish model. Although it is acknowledged that both NETA and NET have similar *in vitro* mammalian primary pharmacology, the secondary pharmacology between these two molecules at high concentrations may differ.

5.3.1 Concentration selection:

The selection of appropriate doses for toxicology studies is an important question. The current draft ICH guidance on reproductive studies ICHS5(R3), with respect to dose, focusses only on mammalian test systems but does provide some useful insights on appropriate dose selection.

Brown et al. chose the concentration range to use in their study based on the concentration range where malformations and embryo lethality was observed in their initial experiments. One method suggested by ICH for predicting the appropriate dose is to consider exposure: '*Doses anticipated to provide an exposure > 25-fold of the clinical systemic exposure at the maximum recommended human dose (MRHD) are generally considered appropriate as the maximum dose for reproductive toxicity studies'.* ICHS5(R3) also states "When the exposure in animals at the NOAEL is >25-fold the exposure at the MRHD, there is minimal concern for the clinical use of the pharmaceutical.

The most conservative estimate for the exposure margin at the NOEC in aqueous media in the Brown et al. study is 42-fold, based on the maternal plasma C_{max} in pregnant women after a single dose of 2 tablets of Primodos (equivalent to NETA 20 mg/EE 40 µg, see **Table 3**). This estimate does not factor in the unbound free drug concentrations, the longer exposure times, the lower fetal distribution demonstrated in mammalian systems, or the high accumulation factor in zebrafish embryos. The Expert Group may wish to consider the concentrations explored by Brown et al. compared to clinical exposures.

Table 3 Exposure margin table based on NET levels as generated by the assessors*.

Nonclinical NETA concentration (µg/mL)	Clinical NET (in Primodos) $Cmax_{total}$ (µg/mL)[a]	Margin based on $Cmax_{total}$ (X-fold)	Clinical NET (in Primodos) $Cmax_{free}$ (µg/mL)[b]	Margin based on $Cmax_{free}$ (X-fold)
1.563	0.074	**21**	0.002	**782**
3.125		**42**		**1563**
6.25		**84**		**3125**
12.5		**169**		**6250**
25		**338**		**12500**
50		**676**		**25000**

[a] $Cmax_{total}$ determined from pregnant women at 6-7 weeks of pregnancy who received a single dose of NETA 20mg:EE 40µg (equivalent of taking 2 Primodos tablets) (Pulkkinen et al., 1984)

[b] $Cmax_{free}$ determined by correcting for binding to sex steroid binding globulin and albumin

* Note, no account is made for the lower (2.5-10-fold) fetal levels vs maternal plasma levels observed in rodents and primates. Also, no account is made for the NETA accumulation in zebrafish embryos demonstrated by Brown et al. under the assay conditions used in their study.

5.4 Accumulation of NETA in mammalian embryos

Brown et al. hypothesize it is possible that the drug concentration will accumulate and build up to high levels in the human embryo over time, as observed in the zebrafish embryos.

Accumulation from a pharmacokinetic perspective relates to the relationship between the dosing window and the rate of elimination of the drug from a compartment. If a dose is not fully cleared from a compartment over time following repeated dosing the levels have the potential to accumulate. In comparison to a mammalian maternal-embryofetal drug distribution system following an oral dose, the zebrafish uptake assay described is a 'closed' system with a constant high drug concentration in the external protein-free embryo media containing 0.2% DMSO. In this system the drug concentration in the external embryo media compartment is expected to remain relatively constant over time as it is in vast excess to the embryo tissue compartment. Under such conditions, an initial absorption phase would be expected followed by a point at which saturation occurs, whereupon the levels would remain constant, depending on the stability of NETA in the embryo and external solution. Any clearance mechanism of the zebrafish embryo may potentially be overwhelmed at high concentrations of NETA. As expected, the study found that high levels of NETA were taken up in the dechorionated 24 hpf embryos within the first 6 hours and these levels were maintained over time.

Published pharmacokinetic studies using radiolabelled NETA in pregnant rats and rhesus monkeys suggest little potential for accumulation of NET in the fetus (Tauber & Humpel, 1984a, Tauber & Humpel, 1984b). Using ^{14}C-radiolabelled NETA following intragastric administration of 1mg/kg NETA and 0.002mg/kg EE in pregnant rats (which on a body surface area estimate is approximately equivalent to a single dose of Primodos), less than 1% of the dose passed the placental barrier (Tauber & Humpel, 1984a). The placenta contained about 20% of the peak maternal serum concentration, measured 30 minutes after administration, whereas the amniotic fluid and fetus contained 11 and 14% of the maternal serum concentration respectively, at this time. At 5 hours following administration, the maternal plasma level dropped to about 5% of C_{max}, with levels in fetus around 6% of the maternal plasma levels. The maximum NET-levels per fetus occurred at 30 minutes post administration and amounted to around 4ng NET/g tissue.

Norethisterone values have also been reported for the rhesus monkey, with maternal plasma levels again being higher than those in the fetus (Tauber & Humpel, 1984a). Pregnant rhesus monkeys given 2 mg NETA/kg plus 0.004 mg EE/kg, intragastrically (equivalent to around 3 times the Primodos dose based on a body surface area estimate), had maternal plasma NET levels of 40 ng/ml after 2 h, which decreased with a half-life of 1.4 h. The fetus contained 0.00083% of the maternal dose of NET and a maximum of around 4-6 ng/ NET/g fetal tissue at 1-2 hrs post administration. The fetal levels are around a maximum of 25% of maternal plasma concentrations with the clearance high-life ($t_{1/2}$) from the fetus only somewhat longer than that in maternal plasma at around 2.1 -2.4hrs (Tauber & Humpel, 1984a). The relative distribution of NET in the fetus was independent of time with about 90% in the carcass at any time, with 3-6% in the liver, and about up to 6% in the brain suggesting no specific enrichment or delayed elimination in the fetal organs.

When reflecting on the Brown et al. hypothesis set out at the beginning of this section (5.4), the Expert Group may wish to consider the pharmacokinetic data obtained from pregnant rats and rhesus monkeys and if extrapolated to pregnant women, do they provide evidence for significant accumulation of NET in the human embryo following exposure of the mother to a single dose of Primodos taken on two consecutive days?

5.5 Risk to humans – calculated safety margins

Determining whether observations in nonclinical studies occur at clinically relevant doses/exposures is key when assessing the translational relevance and risk to humans. The current draft ICH guidance on reproductive studies ICHS5 (R3) states that “Comparison of pharmaceutical exposure at the No Observable Adverse Effect Level (NOAEL) in the test species to that at the MRHD (Maximum Recommended Human Dose) is a critical determination”. It further states, “When the exposure in animals at the NOAEL is > 25-fold the exposure at the MRHD, there is minimal concern for the clinical use of the pharmaceutical”.

In the study by Brown et al., no developmental effects were seen in zebrafish embryos at a concentration of 3.125 µg/mL NETA + 0.00625 µg/mL EE. Taking the most conservative approach the exposure margin relative to humans is 42-fold based on maternal $C_{max_{total}}$ plasma concentration following 2 doses of Primodos. There is no documented protein in the zebrafish assay medium and thus if the available unbound free drug fraction is considered, the exposure margin increases to 1563-fold (see **Table 3**). In rats and non-human primates, fetal levels were reported to be 10 - 25% of maternal plasma levels and so, depending on how well the data extrapolate to humans, the calculated margins could be at least 4-fold greater again. The Expert Group may wish to consider the exposure margins determined in this study in the context of ICH guidance.

At a concentration of 12.5 µg/mL NETA + 0.025 µg/ mL EE, most surviving embryos displayed one or more malformations. Under the experimental conditions used, significant accumulation (≥40-fold accumulation ratio) of NETA occurred in the zebrafish embryo and levels of NETA per embryo after 6 - 48hrs of incubation are equivalent to around 500-900 µg/g wet tissue. By comparison the maximum levels of NET in rat fetuses following administration of the equivalent of a single Primodos dose are significantly lower, at around 0.004 µg/g wet tissue and are therefore many orders of magnitude (>100,000-fold) lower than those in zebrafish embryos where developmental effects take place (**Table 4**). Similar maximum fetal levels (0.004 – 0.006 µg/g wet tissue) to those in rats were obtained in studies in rhesus monkeys following the equivalent of around 3 times a single Primodos dose. The Expert Group may wish to consider the differences in the maximum fetal levels between the zebrafish and mammalian studies and the potential clinical relevance in relation to the observed developmental effects.

Table 4. Maternal and embryo fetal levels of NET/NETA in pregnant women, rat and rhesus monkeys compared to zebrafish (human data Pulkkinen et al., 1984, rat and primate data from Taber & Humpel, 1994a and zebrafish data from Brown et al., 2018).

Species	NETA dose/ concentration	NET/NETA maternal plasma C_{max} / zebrafish embryo media ng/ml (Tmax h)	Maximum embryofetal NETA level ng/ g wet weight tissue (time h)
Pregnant women	20mg (~0.4 mg/kg)	74 (3 h)	not determined
Pregnant rhesus monkey	2 mg/kg	41 (2 h)	4-6 (1-2 h)
Pregnant rat	1 mg/kg	31 (0.5 h)	4.1 (0.5 h)
Zebrafish	12.5 µg/ml	12,500 (0-72 h)	900,000 (24 h)

The Expert Group may also wish to consider the implications of the short gestation period of the zebrafish embryos, where effects were observed following constant high levels of drug for periods ranging from 1 to 72 hours, compared to the human setting in which women received one dose of NETA/EE (10mg/20µg) on each of two consecutive days.

6. Advice sought

On the basis of the evidence considered, the ad hoc Expert Group is asked to advise on;

- the suitability of the zebrafish model for evaluating effects of norethisterone and ethinylestradiol in human pregnancy;
- the robustness of the Brown et al. study; and
- any clinical implications.

7. Summary

In the light of the results and in the context of the current international guidance the Expert Group may wish to consider the advantages and limitations of the study with regard to the following.

- Both the advantages and potential limitations of zebrafish as a model organism for studying norethisterone and ethinylestradiol in human pregnancy considering the lack maternal influences, placental function and the role of sex steroids in the establishment and maintenance of human pregnancy

- Differences in species receptor pharmacology in zebrafish compared to humans
- Differences in the route of administration, timing and duration of exposure compared to clinical exposures
- The concentrations of NETA achieved in the zebrafish embryo compared to the exposure levels achieved in mammalian studies

John Clements
Ross Hawkes
7th September 2018

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