18 October 2018
EMA/745160/2018
Committee for Medicinal Products for Human Use (CHMP)

Assessment report

Procedure under Article 5(3) of Regulation (EC) No 726/2004

INN/active substance: norethisterone and ethinylestradiol

Procedure no: EMEA/H/A-5(3)/1470

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1. Information on the procedure

Recently available data was brought to the attention of the competent authorities suggesting that a combination of ethinylestradiol and norethisterone may cause embryonic damage in a zebrafish model in a dose and time responsive manner. Since ethinylestradiol and norethisterone are commonly found in a range of widely-used authorised gynaecological medicinal products across the European Union (EU), there is significant public health interest that the Agency provides a scientific opinion on the interpretation of the new study (Brown *et al*., 2018) which concludes that these substances are potentially teratogenic. Indeed, whilst these substances are contraindicated in pregnancy, studies show that a substantial number of women continue to take them without realising they are pregnant.

At its May 2018 meeting following a request by the MHRA (UK), the CHMP therefore initiated a scientific review of this study, to give a scientific opinion on the zebrafish model for evaluating effects of norethisterone and ethinylestradiol in human pregnancy, the robustness of this study and on any potential clinical implications of the results presented on the human foetus.

2. Scientific Discussion

2.1. Introduction

The synthetic oestrogen ethinylestradiol (EE) is the oestrogen component in all combined hormonal contraceptives (CHCs) that are available on the market, except for two CHCs which contain estradiol valerate. The dose of ethinylestradiol in CHCs ranges between 0.015 – 0.035 mg. Contrary to the oestrogen component, the progestogen component varies in CHCs. There are several different synthetic progestogens including norethisterone acetate (NA). The norethisterone dose in CHCs ranges between 0.5 and 1 mg.

Synthetic progestogens are derived from 19-nortestosterone or from progesterone. The mechanism of action of a progestogen is to suppress the secretion of luteinizing hormone (LH), which will prevent ovulation, while the oestrogenic component suppresses the secretion of follicle stimulation hormone (FSH), which will prevent the development of a dominant follicle.

Primodos was a hormonal pregnancy test which was available on the market in the UK between 1958 and 1978. The product contained norethisterone acetate (NA) 10 mg + ethinylestradiol 0.02 mg. the dosing scheme was one oral tablet for two days.

Norethisterone and several other progestogens are also used as monotherapy or in combination with an oestrogen for the treatment of menstrual bleeding irregularities and endometriosis. The use of norethisterone and other progestogens in the treatment of menstrual irregularities and endometriosis is based on antagonistic effect on oestrogen-mediated endometrial growth during the menstrual cycle. Norethisterone is used for menstrual irregularities in a dose range of 5 - 15 mg and as monotherapy for endometriosis in a dose of 10 mg/day.

2.2. The zebrafish as a model for evaluating effects of norethisterone acetate and ethinylestradiol on human pregnancy

The zebrafish as a model for developmental toxicity

The use of the zebrafish as a biological model system for toxicology research focusing on emerging trends was recently reviewed by Horzmann and Freeman (2018). Zebrafish embryos develop rapidly *ex*

utero with all major systems formed by 72 hours postfertilisation (hpf). The development is highly conserved among vertebrates and the optical transparency of zebrafish embryos permits visual analyses of early developmental processes and organ morphology offering an attractive alternative assay for quick screening of embryotoxicity and teratogenicity. It was suggested that the response of zebrafish to developmental toxicants is on par with mammalian models of toxicity which supports the use of the zebrafish model in toxicology research.

The zebrafish model is currently used by pharmaceutical developers as a screening assay for detecting developmental toxicants (Sipes *et al.,* 2011; Gustafson *et al.,* 2012; Ball *et al.,* 2014; Beekhuijzen *et al.,* 2015; Nishimura *et al.,* 2016). Zebrafish embryonic development can be followed relatively easily microscopically due to the transparency of the eggs, and multiple transgenic reporter gene zebrafish models have been developed to study mechanisms of action in development and developmental toxicity. The zebrafish model is a relatively high-throughput method compared to the *in vivo* rat and rabbit developmental toxicity studies. Furthermore, it has been shown that in the critical part of development (gastrulation), during which the vertebrate embryo is most susceptible to adverse effects from xenobiotics, gene expression profiles among vertebrates are mostly conserved (Irie and Kuratani, 2014). Many different protocols have been developed over the years and are applied by academia and industry, so currently there is no uniform protocol for assessing developmental toxicity in zebrafish embryos. However, there have been proposals for technical harmonization, taking into account the state-of-the-art knowledge. These attempts at establishing a best practice often include a point-based morphological scoring.

The updated ICH S5(R3) guideline does not mention the Zebrafish embryotoxicity test (ZET) in the finalised concept paper or in the Step 2 draft version published in July 2017. In principle the draft guideline does not recommend specific assays but includes basic principles to assist in assay qualification for potential regulatory use.

Whilst alternative assays as alternative approaches for developmental toxicity testing are under development in order to replace the standard rodent and non-rodent developmental toxicity studies to a certain extent in specific scenarios of use, such as specific indications (ICH S5(R3) step 2, 2017), for most indications, a rodent and/or non-rodent developmental toxicity study will remain mandatory. It is expected that variants of the zebrafish developmental toxicity assay, probably in combination with other alternative approaches for developmental toxicity, will be applied for qualification in the future. However, no zebrafish developmental toxicity test has currently been qualified or validated for regulatory testing of pharmaceuticals.

A drawback of the zebrafish embryo model is that the zebrafish is not a mammal, and does not provide information on aspect related to mother-to-foetus interactions. Therefore, maternal pharmacokinetics and secondary effects on the embryo caused by maternal pharmacology and toxicity are intrinsically absent from the currently proposed zebrafish models. In addition, zebrafish are cultured in water, which provides a different matrix for compounds compared to mammalian plasma and should be taken into account when extrapolating exposure between fish and mammal/human.

There is currently no validated protocol or guidance for performing a zebrafish embryo toxicity assay. The closest guideline is the OECD 236 fish embryo acute toxicity test (FET) with the zebrafish (*Danio rerio*) embryo which is used in ecotoxicology risk assessments (OECD, 2013). In this protocol, newly fertilised zebrafish eggs are exposed to the test chemical (20 embryos per concentration, one embryo per well in 24-well plates) for a period of 96 hours. Every 24 hours, lethality is recorded by observing coagulation, lack of somite formation, lack of detachment of the tail-bud from the yolk sac, and lack of heartbeat.

Several research groups have published attempts to develop the OECD 236 protocol or similar assays by adding developmental and teratogenicity end-points for the purpose of human and environmental risk assessments of chemicals and pharmaceuticals. A thorough analysis of published peer-reviewed manuscripts on Zebrafish embryotoxicity testswas done by Beekhuijzen and colleagues (2015) and the most optimal test conditions, including a morphology scoring system, were proposed. Their proposal is based on the OECD 236 fish embryo acute toxicity test test with modifications and additions based on the performed review. The use of 30 embryos per concentration was recommended for assessment of teratogenicity as more variability can be seen with this endpoint compared to mortality. It was also pointed out that the concentrations being tested should not cause lethality, or at least at maximum 10 % acute mortality at the highest test concentration. The use of DMSO as a solvent was also discussed and the recommendations were to use a maximum of 0.1 % (v/v), or possibly up to 0.5 % (v/v) with a doubling of the number of exposed embryos. Exposure should start at 4 hpf (corresponding to implantation in rats and rabbits) and end at 100 hpf (96 hours exposure) with daily observations on morphology. The chorion should be left intact to be able to detect effects on hatching. Dechorionation could even lead to malformations if automated dechorionation at 4 hpf was used. Internal exposure should be verified by bioanalysis in order to detect possible false negatives or false positives. False negative results have been reported with a known human teratogen due to very low internal exposure, and vice versa false positive results have been reported where bioanalysis revealed a very high uptake.

The proposed morphology scoring system includes both general developmental delay and teratogenicity. Observed effects related to developmental delay include: detachment of the tail, somite formation, eye development, movement, circulation, heartbeat, pigmentation of the head and body, pigmentation of the tail, yolk extension nearly empty, pectoral fin, protruding mouth. Malformation of the head, malformation of the saccule/otholiths, malformation of the tail, malformation of the heart, deformed body shape, and yolk deformation are considered related to teratogenicity. The authors suggested determining a benchmark dose (BMD) for developmental delay at 24, 48, 72 and 96 hours and teratogenicity (one BMD for number of embryos with malformation and one for total number of malformations per embryo). However, as the authors pointed out, more research is needed to define the threshold value to be used for teratogenicity classification.

A detailed description of methods to assess lethality and malformations during zebrafish development was also recently published as a book-chapter (Raghunath and Perumal, 2018). Two major malformations scoring systems to assess the malformations in zebrafish embryos/larvae were proposed: binominal and relative morphological scoring systems. Zebrafish housing and breeding, embryo collection and care and embryo culture and testing conditions are described. Briefly, at least 30 embryos per concentration is recommended for lethality and teratogenicity testing. The use of 24-well plates is recommended over the 96-well plate to provide more oxygen and to allow more swimming space. The concentrations considered for the test should not cause lethality. For teratogenicity testing the exposure should cover the period of zebrafish development. Exposure should start at 4 hpf and must cover 96 hpf but not extend beyond 120 hpf.

A scoring system according to Panzica-Kelly and colleagues (2010) is described. The malformations are analysed only for dechorionated embryos exposed from 4 hpf until 5 dpf. The embryos/larvae are anaesthetized and assessed under stereomicroscope. Observation of craniofacial structure, upper and lower jaws, pharyngeal arches, upper facial structures, brain, heart morphology, notochord, somites, body shape, fins, tail are made and scored on a scale from 0.5 to 5. To evaluate the development on an hourly or daily basis with intact chorion the extended Hermsen's general morphology scoring system is instead proposed. The heart rate, pericardial sac area, heart tube looping, and atrioventricular rhythmicity are measured quantitatively. Cartilage and bone malformations are visualised and scored in a semi-quantitatively way. An assessment of malformations such as pericardial

oedema, yolk sac oedema, body axis bent, malformation of the head, malformation of the eyes, oedema of the eye/head, pigmentation in the eye, malformation of saccule/otoliths, malformation of tail, malformation of heart, circulation, modified chorda structure, movement, pigmentation in the head and tail, and scoliosis is made and scored either as presence or absence (1 or 0) or in a relative degree (0-absence, 1- slight variation, 2- mild, 3- moderate, 4- severe).

The calculation of a teratogenicity index (TI) as either the ratio between the LC_{50} and the EC_{50} (TI >1), or as preferred by the authors, the LC_{25} and the NOAEL (TI > 10), is proposed for classification of the compound as a teratogen.

None of the above described methods has been validated or qualified for regulatory use for pharmaceuticals.

Suitability of the zebrafish for testing norethisterone acetate (NA) and ethinylestradiol (EE)

The main target receptors for NA/NE (Nuclear Progestin Receptor (PGR)) and EE (oestrogen receptor, ER) have been described, characterized and functionally analysed in the (developing) zebrafish (Hanna *et al.,* 2010; Tingaud-Sequeira *et al.,* 2014; Schaaf, 2017). However, these receptors are structurally different from human. In human, as in most vertebrates, two PGR proteins (PGR-A and PGR-B) have been reported, which are derived from a single locus but have a different length, in that PGR-B (114 kDa), in contrast to PGR-A (94 kDa), has an additional 165 amino acids present in the N-terminus. PGR-A and PGR-B have a partly overlapping structure, but function as separate transcription factors with different physiological actions and gene regulation. ERa downregulation, for instance, appears to be PGRB-specific. In zebrafish, in contrast to human, only one single PGR (68 kDa) has been described, which has, compared with the human PGR, an 89% homology in the DNA binding domain and only a 68% homology in the ligand binding domain. The low homology is in line with phylogenetic analysis, which indicated that zebrafish PGR clustered together with those of other fish species, and distinct from tetrapods and mammals (Hanna *et al.,* 2010). The zebrafish PGR protein has been found abundantly in ovaries, testis, and brain and shares with mammals the binding affinity for progesterone but also, unlike for mammals, the higher affinity for the fish specific progestin 17,20β-DHP (Pinter and Thomas, 1997). In this respect it is important to mention that larger PR subtype, PGR-B, which is lacking in zebrafish, forms heterodimers with the PGR-A, has an additional AF3 activity, activates other genes than PGR-A and even opposes the PGR-A activity. (Jacobsen and Horwitz, 2012).

With respect to the oestrogen receptor (ER) two subtypes, ERα(esr1) and ERβ(esr2) have been found in mammals, but teleost, like zebrafish, also have not one but two ERβ genes (esr2a and ers2b), phylogenetically related to tetrapod ERβ. It has been shown in mammals that ERα and ERβ have different hormonal specificity and tissue distributions. In zebrafish, mRNAs of he three types of ER largely overlap and are predominantly expressed in brain, pituitary, liver, and gonads. Compared to human ER's an 92% & 91% homology in the DNA binding domain was found but only a 55% & 57-61% homology in the ligand binding domain, for ERα & ERβ's subtypes respectively. Phylogenetic analysis suggests that zebrafish ERα is derived from ancestral ERα but suggests that the teleost ERβ family is more complex than that of mammals, raising the question of the specific function of this additional ERβ subtype. All subtypes bound oestradiol *in vitro* at sub nM concentration like mammalian ER's, but the transactivation activity of ERβ's seemed to be stronger in zebrafish (Menuet *et al.*, 2002).

Taken together, given the differences in PGR and ER subtype expression, ligand binding and tissue distribution, it is unclear if their role in physiology is comparable between zebrafish and human.

The zebrafish embryonic developmental toxicity model and extrapolation to effects on human pregnancy

As for all models for developmental toxicity, direct extrapolation of specific developmental defects or defects in a specific organ between species, including human, remains difficult for all models of developmental toxicity. In a retrospective study, rat and rabbit developmental toxicity data of 378 pharmaceuticals were compared and it was found that in approximately 80% of these pharmaceuticals, rat and rabbit both gave the same outcome for the absence or presence of developmental toxicity (Theunissen *et al.,* 2016). However, in only a fraction of these studies, effects on development of the same organs were observed (Theunissen *et al.,* 2016). In addition, specific organs affected could even differ between strains of a species (Janer *et al.,* 2008; Theunissen *et al.,* 2016). Though, when a compound induces developmental defects in the same organs across species, this may provide an increased concern for exposure during human pregnancy (ICH, 2017; EMA Guideline on Risk Assessment of Medicinal Products on Human Reproduction and Lactation: From Data to Labeling, 2009).

In the step 2 document of the ICH S5(R3) revised guideline, it is stated that animal tests for developmental toxicity only have to be performed up to 25-fold the safety margin (e.g. comparing maternal blood concentration at the developmental NOAEL in rat or rabbit to the exposure at the maximum recommended human dose (MRHD)) (ICH, 2017). The rationale behind this is that for all known human teratogens the safety margin was less than 25-fold in rat and rabbit. Therefore, exposures tested orders of magnitude higher than the 25-fold margin are not likely to be relevant for the human situation. This may also be applicable for alternative test systems, such as those examining zebrafish developmental toxicity.

Conclusion on the suitability of the zebrafish model for evaluating effects of norethisterone and ethinylestradiol in human pregnancy

The zebrafish is a well-established model to screen for compound induced adverse effects on embryonic development. However, no zebrafish developmental toxicity test has currently been qualified or validated for regulatory testing of pharmaceuticals. Receptors targeted by NA and EE have been described, characterized and functionally analysed in the zebrafish. However, between zebrafish and human, structural and functional differences between these receptors exist, and therefore it is unclear if their role in physiology is comparable between zebrafish and human. Developmental defects observed in animal studies, including the zebrafish model, cannot be directly extrapolated to possible effects on human pregnancy. When multiple animal models/species show similar phenotypic effects, this increases concern in relation to a potential human relevance. If qualified, a well-performed zebrafish developmental toxicity test may contribute to the evaluation of the teratogenic potential of a compound as part of an integrated testing strategy.

For extrapolation to the human situation, the zebrafish should be seen as complimentary to the currently available animal tests. A proper qualification of a zebrafish developmental toxicity test has not yet been performed and it is premature to conclude on its suitability to predict potential teratogenic effects of norethisterone and ethinylestradiol in human pregnancy. The results of such a study still needs to be evaluated together with all available *in vivo* non-clinical and human data, including exposure data, as part of an integrated risk assessment approach.

Norethisterone acetate and ethinylestradiol in other non-clincial models

The CHMP noted that an in-depth review (CHM, 2017) of available non-clinical data on the reproductive and developmental toxicity following exposure to NA and EE was published as annex (Annex 20)[1] to the Report of the Commission on Human Medicines' Expert Working Group on Hormone Pregnancy Tests.

The findings and conclusions of that review are briefly summarised here. For synthetic progestogens related to NA, embryo lethality and genital malformations of the foetus are the main findings at doses generally higher than those used in the hormone pregnancy tests. This finding was observed in a number of different animal models including mice, rats, guinea pigs, rabbits and non-human primates. The mechanism of embryo lethality is not known. For NA, no clear evidence for non-genital teratogenicity has been reported in any species.

For estrogens, such as EE, given at high doses during pregnancy in rodents and rabbits, embryonic death is reported and reflects the established abortifacient effect of these compounds. Genital tract abnormalities have been reported following continuous exposures of doses higher than those used in the hormone pregnancy tests. No evidence of an increase in malformations in non-reproductive tissue following EE exposure was reported.

For the combination of synthetic progestogen and estrogen, embryolethal effects were consistently observed when dosed through early gestation and occurred at daily doses similar to or higher than those used in hormone pregnancy tests. Genital malformations have been observed at high doses in some of these studies.

The majority of studies with NA and EE suggest that non-genital malformations do not occur. In one study in mice exposed to NA and EE throughout organogenesis, an increase of visceral malformations was reported at a dose that was also embryo lethal and higher than those used in hormone pregnancy tests. Lower doses did not produce an increase in malformations.

In some studies with NA and EE and other combinations at higher doses in rats and rabbits there was evidence of an increase in developmental variations. These findings provide evidence of embryofoetal toxicity but not of teratogenicity. In non-human primates exposed to NA and EE combinations, similar findings of embryo lethality and genital abnormalities without any increase in non-genital malformations were reported.

Overall, it was concluded by the CHM (2017) that there is no clear evidence that a combination of NA and EE administered during pregnancy in animal studies causes malformations in non-reproductive tissue.

2.3. The assessment of the experiments by Brown and colleagues (2018)

The publication contains a number of *in vitro* studies investigating the effects of a combination of NA and EE, administered as a single-dose with exposure up to 48 hours, on zebrafish embryo development and survival. The duration of dosing was designed to be comparable to the one to two days dosing regimen used for Primodos. In addition, mechanistic studies were performed on effects on blood vessel formation in zebrafish embryos and in human umbilical vein endothelial cells as well as effects on neurite outgrowth in zebrafish embryos and in mouse retinal explants. The ratio of NA/EE were fixed at 500:1 which is equivalent to the ratio of the formulation of Primodos (10 mg NA and 0.02 mg EE).

[1] Review of non-clinical evidence of reproductive and developmental toxicity for norethisterone acetate and ethinylestradiol (https://mhra.filecamp.com/public/files/2ou7-p1dlcbo2).

Effects on zebrafish embryo (wildtype) development and survival

Concentration-response relationship

The concentration-response relationships on survival rate and gross defects rate were investigated using 6 concentrations of the NA/EE mixture (1.563 µg:3.125 ng/mL; 3.125 µg:6.25 ng/mL; 6.25 µg:12.5 ng/mL;12.5 µg:25 ng/mL; 25 µg:50 ng/mL; 50 µg:100 ng/mL). NA and EE were dissolved in DMSO and diluted in distilled water. Wildtype zebrafish embryos at 24 hpf were hand dechorionated and exposed to the compound or the vehicle (DMSO at 0.2 %) and incubated for 48 h, until 96 hpf. Detailed analysis of the effects on embryogenesis was performed by measuring the overall size of the embryos, pectoral fin size, otic vesicle size and eye size.

There was a concentration-dependent decrease in survival rate starting at 6.25 µg:12.5 ng/mL. The manuscript states a 10 % increase in embryonic death whilst the corresponding figure is rather showing a 20 % decrease in survival rate at this concentration. At 25 µg:50 ng/mL and higher 100 % embryolethality was observed. There was also a concentration-dependent increase in gross defects starting at 6.25 µg:12.5 ng/mL. A discrepancy between the manuscript, stating a 20 % increase, and the corresponding figure, showing a 10 % increase is noted. At the next concentration 43 % embryolethality and 92 % increase of gross defects were observed.

At 6.25 µg:12.5 ng/mL the overall body length and the length of the pectoral fins were decreased. At 12.5 µg:25 ng/mL overall body length, length of pectoral fin, size of the eye and size of the otic vessel were decreased by 15-17 % compared to the DMSO controls. At the high concentration bent spine, pericardial and yolk sac oedema and oedematous yolk sac extension was also observed. The conclusion from this experiment was that the NA/EE mixture impairs development in a dose-dependent manner.

The number of embryos exposed per concentration, number of replicates, or number of embryos per well was not stated. In the results section n≥ 15 per treatment is stated whereas in the corresponding figure n-values ranging from 7 to 13 are indicated. It is further not clear why these n-values are different for the same treatment group for the different end-points. Methods used to qualitatively and quantitatively evaluate and score lethality and gross defects were not reported. The number of affected embryos per concentration and number of observations per embryo was not reported. The background incidence of the observed morphological defects in the used zebrafish strain was not reported.

It was also noted that neither a negative control (dilution water only) nor a positive control was included.

Despite these shortcomings it may be concluded that direct exposure of a mixture of NA/EE, at high concentrations for 48 hours, impairs survival and development of dechorionated zebrafish embryos. The nominal no observed effect concentration (NOEC) for both survival rate and gross defects rate was 3.125 µg:6.25 ng/mL.

Age-dependence of embryos

In a subsequent experiment embryos (n > 15 for all time points and concentrations) at different ages (6, 24, 48 and 72 hpf) were exposed to the 12.5 µg:25 ng/mL concentration for 24 hours and then fixed and imaged.

Embryos at 6 hpf exhibited severely malformed tails and bent spines, malformed pericardial sacs, yolk sac damage/oedema and very small eyes. Embryos exposed at later time points had less severely bent spines, mild pericardial defects and apparently normal eyes and otic vesicles. The only consistently observable issue was an oedematous yolk sac.

Exposure time-dependence and effects on movement

Twenty four (24) hpf embryos were exposed to the 12.5 µg:25 ng/mL concentration for differing time periods from 1 to 24 hours and overall body length, eye area and yolk sac was measured. The first morphological damage was evident from 4 hours after NA/EE mixture application.

In addition, the movement of embryos was determined by time lapse recording for 2 minutes and counting the number of time each embryo moved. Embryos (24 hpf) were exposed to 12.5 µg:25 ng/mL mixture in 24-well plats (no more than 5 embryos/well) for 1-24 hours. Following one hour exposure the average number of movements per embryo per minute was 16 for DMSO treated embryos compared to the average 0 for the NA/EE treated embryos. Inhibition of movement was also observed at all time-points assessed up to and including 4 hours of exposure (n≥ 5).

It is again not clear how many embryos were exposed/analysed per time and treatment. In the presented figures n-values ranging from 5 to 15 are indicated and again it is not clear why these n-values are different for the same treatment group for the different end-points. Methods used to qualitatively and quantitatively evaluate and score morphological damage, including severity grading, were not reported. The number of affected embryos and number of observations per embryo is not clear.

For the movement analysis the number of embryos was 5 in the DMSO group and 4 in the treated group at 1 hour (stated in figure text). It is noted that all embryos in each group were exposed and filmed together in a single 24-plate well. It is not clear if there were any replicates or if the filming was repeated over more than one 2 min period. It is also not clear if the movement analysis was included for the same embryos as the ones analysed for morphological damage.

It may be concluded that early stage zebrafish embryos are more sensitive to the NA/EE mixture than later stage embryos. It was also shown that in addition to lethality and gross defects, inhibition of movement was induced by exposure to the NA/EE mixture, however the reproducibility of this finding is unclear.

Quantification of the dose of the drug that reaches the embryo

The concentration of NA in 24 hpf embryos exposed to the 12.5 µg:25 ng/mL NA/EE mixture or DMSO or water for 6, 24 or 48 hours was measured using a LC-MS/MS assay. Following exposure the embryos were rinsed in water three times to remove excessive solution. Embryos were stored individually in 100 µl of water and frozen before analysis. Individual embryos in 100 µl of water were homogenised by sonication and the resulting solution diluted, centrifuged and 5 µl was injected onto the chromatograph. The levels of NA in the embryos were 1077±19.05 ng/mL (n=5) at 6 hours, 1872 ± 71.65 ng/mL (n=19) at 24 hours, and 1162 ±22.57 ng/mL (n=4) at 48 hours. NA was not detected in the water and DMSO controls at any time point.

The validity and accuracy of the analysis method were not present in the publication and therefore not been assessed. The LLOQ for the assay is stated to be 1 ng/ml and the intra and inter-assay variation determined to be < 3% and < 6 % respectively.

It is not clear if the embryos were exposed in single-wells or together. The initial volume of test solution/exposure media was not stated. The number of analysed embryos varies from 4 to 19 per treatment. It is not clear why there is such a large difference in number of analysed embryos per treatment group.

Exposure data is presented as ranging from 1 to 1.8 µg NA/embryo in the text, whereas in the corresponding figure and table the exposure data is presented as a concentration, ranging from 1077

to 1872 ng/mL. Other data of importance such as weight of the embryos, growth rate, or volume of embryo + dilution water, weight of pellet and volume of supernatant following centrifugation or any other parameter that could relate the amount of detected NA to amount of biomass was not presented.

It is noted that no data on uptake and exposure of EE was presented, only a brief statement that levels of EE were consistently below detection rates.

The data indicates that there is an uptake and internal exposure of NA in the zebrafish embryos. However, the data is limited and no conclusions on bioaccumulation potential can be made.

Effects on cell death and cell proliferation

A TUNEL assay, quantifying the number of apoptotic cells, was performed on zebrafish embryos (24 hpf) exposed to the 12.5 µg:25 ng/mL NA/EE mixture or DMSO and fixed at 6 or 24 hours after exposure (n≥ 5 for each condition and time point). At both time-points cell death was increased in the NA/EE exposed groups. The increased cell death was reported not to be localised to specific tissues but was observed throughout the embryos, correlating with the decrease in overall body size, fin and eye size. In addition, antibody staining with anti-Phosphohistone H3, quantifying the number of mitotic cells, was performed in an identical experimental set-up (n= 11 for 6 hours, n= 16 for 24 hours in NA/EE exposed embryos). Embryos treated with NA/EE presented a lower number of proliferating cells in all regions of the embryos at 6 and 24 hours, compared to the DMSO controls.

The TUNEL assay was performed using a commercial *in situ* Cell Death Detection Kit (TUNEL-Roche).

The results show that the NA/EE mixture at 12.5 µg:25 ng/mL causes a general cell death and a decrease in cell proliferation in zebrafish embryos following 6 and 24 hours exposure.

Mechanistic studies

Effects on blood vessel formation

The transgenic *fli1*:EGFP reporter line of zebrafish embryos at 24 hpf were exposed to the 12.5 µg:25 ng/mL NA/EE mixture (n= 19 at 6h, n=18 at 24 h) or DMSO (n= 18 at 6h, n=21 at 24 h) and their intersomatic vessels were imaged after 6 or 24 hours exposure. Incubation with the NA/EE mixture caused some mispatterning of vessels within 6 hours exposure and misplacing, mispatterning and stunting of intersomitic vessels outgrowth throughout the spine of the embryo 24 hours following drug exposure. Quantification of intersomitic vessel outgrowth demonstrated no outgrowth deficit at 6 hours but significant reduction in outgrowth by 24 hours.

The effect of the NA/EE mixture was also tested using *in vitro* cultures of the human umbilical vein endothelial cell line (HUVEC). Effects on number of branches of endothelial tubes, cell proliferation and cell number were observed. The cells were still able to form patterned, branched, vascular networks.

Effects on neurite outgrowth

The effect of the 12.5 µg:25 ng/mL NA/EE mixture on neurite outgrowth in 24 hpf wildtype zebrafish embryos following 6 or 24 hours (n=9 at 6 hours, n=16 at 24 hours) exposure was investigated. Following exposure the embryos were fixed and stained with an anti-neurofilament antibody to analyse nerve patterning. Embryos treated with the NA/EE mixture presented defasciculation of axons and shortening of axonal outgrowth. Quantification of nerve length relative to overall body length indicated significant nerve length reduction from 6 hours of exposure. In the developing head of the embryo nerves were also disorganised, mispatterned and defasciculated when compared to DMSO exposed embryos.

The effect on neurite outgrowth was also investigated in an *in vitro* mouse retinal explant assay. Retinas were dissected from E14.5 C57BL/6J WT mice and cultured in DMSO or in a range of concentrations of the NA/EE mixture. After 48 hours the cultures were fixed and stained with a neuron-specific anti-β-tubulin antibody and the area of neurite outgrowth was quantified. The results showed a dose-dependent inhibitory effect on neurite outgrowth starting at the 6.25 µg:12.5 ng/mL NA/EE concentration.

The fact that the NA/EE exposed HUVEC cells were able to form patterned, branched, vascular networks indicates that NA/EE does not have strong anti-angiogenic properties. The observed general effects on cell survival and proliferation are a plausible cause to the observed effects on blood vessel formation and neurite outgrowth.

Clarification provided

Additional information was kindly provided by the corresponding author. It could be clarified that the zebrafish embryos were exposed to the NA/EE mixture in 24-well plates with 5 embryos per well. The volume of exposure media was 0.5 ml per well. The experiments were repeated more than 5 times by several investigators. The scoring of morphological defects was binomial scoring of structure/malformation present or not present. The unit for the reported NA concentrations in the zebrafish embryos is µg/embryo.

The methods used was said to be mainly based on the methods described in Parng *and colleagues* (2002) and this publication was provided, together with an updated written description of the methods.

It was also clarified that the increased cell death was reported not to be general but localised to specific tissues (not detailed which specific tissues). Although in the manuscript it was stated repeatedly that cell death was "*not localised to specific tissues, […] but was observed throughout the embryo*".

The information regarding the repetitions of the experiments would explain the confusing n-numbers for the different exposure groups. However, it remains unclear why the number of controls was very low, specifically for the concentration-response experiment. According to the author, consistent results were obtained indicating that the results are reproducible.

In the provided manuscript, the use of zebrafish bioassays for assessing toxicity, angiogenesis, and apoptosis is described. However, there is no detailed description of the zebrafish embryotoxicity and development test. It is unclear whether the zebrafish embryos were cultured and exposed in 96-well microtiter plates rather than 24-well plates. Besides the determination of the LC_{50} the main focus is on the staining methods to visualise angiogenesis and apoptosis in order to facilitate high-throughput screening of drugs using a microplate reader. There is no description on determination or scoring of teratogenicity end-points.

In conclusion, some of the questions regarding culture conditions were clarified; however, detailed descriptions are still lacking and there is conflicting information taking into account the description provided in Parng and colleagues (2002) as well as the information on general or localised cell death.

This publication presents a number of experiments of exploratory nature on the effects of a mixture of NA and EE on the survival and development of zebrafish embryos at different stages. In addition, *in vitro* studies were performed on cell survival and proliferation, blood vessel formation, and neurite outgrowth, presumably to test hypotheses on mechanisms contributing to the observed effects.

The zebrafish studies was not claimed to be performed in accordance with any validated protocol or guidance and it is clear that it was not performed in accordance with the above described proposal(s)

for a zebrafish embryotoxicity study. The descriptions of the methods included in the publication are in several cases succinct and numerous pieces of information crucial for the assessment of the reliability of the tests and interpretation of the results are missing. The reliability, accuracy and reproducibility of the results can therefore not be fully assessed.

It is noted that if the reliability of the study for use of the data in an environmental risk and/or hazard assessment was to be evaluated using the Criteria for Reporting and Evaluating ecotoxicity Data (CRED) or the Klimisch method (Moermond *et al.,* 2016, Kase *et al.,* 2016) this study would most likely be assigned as "R4: not assignable".

Method description

The intended design of the studies, including statistical methods, was not described. Information such as initial number of embryos per exposure group, initial number of embryos per well, the use and performance of internal plate controls and the number of replicates was not presented. The size and material of the incubation plates and the volume of exposure media, or if any cover such as lid or self-adhesive foil was used, was not reported. Methods used to qualitatively and quantitatively evaluate and score lethality and morphological damage, including severity grading, were not reported. Some clarifications were provided by the corresponding author but there are still inconsistencies and unclarified issues remaining.

Validity and control

The use of any validity criteria was not reported. For instance, the FET performed in zebrafish embryos according to OECD 236 requires that overall fertilization rate of eggs should be ≥ 70% in the batch tested, water temperature should be maintained at 26 ± 1°C in the test chambers, overall survival in dilution-water and solvent control should be ≥ 90 % at 96 hours, the dissolved oxygen concentration in the negative control and the highest test concentration should be ≥ 80 % at 96 hours. In addition, the hatching rate in the negative control should be ≥ 80 % and a positive control should be used and should result in a minimum mortality of 30%. At least some of these criteria would be applicable also for a zebrafish embryotoxicity study.

It is not stated if loss of exposure media during the test through evaporation was controlled. Incubation temperature, pH, oxygen saturation and control of these parameters were not reported.

The solubility and stability of the test compounds in water was not reported. Any confirmation of the nominal concentrations in the stock solutions or in test chambers before and at termination of the studies was not reported.

For internal validation of *in vitro* experiments, positive and negative control substances should be taken into account in each test run (OECD, 2018). A solvent control, DMSO at 0.2 %, was used. However, a negative control (dilution water only) was not used except for in the study analysing the NA concentrations in the exposed embryos. Positive controls were also not used.

Presentation of the results

The resulting data is not presented for all concentrations or time points investigated in the different experiments. Survival rates and number of analysed embryos in the individual tests were not reported. The number of affected embryos and number of observations per embryo per test is not reported. Moreover, there are discrepancies in the data presented in the text of the manuscript and the corresponding figures.

Interpretation of the results

It should be noted that the individual components were not tested separately in any of the experiments. Therefore, the interpretation of the results is limited to the mixture as such in the fixed ratio of 500:1. Consequently, it is not possible to discern how each of these compounds influenced the outcome in the mixture experiments. Therefore, it is not possible to provide a conclusion on separate effects of these two compounds on zebrafish development. This information would have been valuable to interpret the results of the manuscript, especially as the EE dose in Primodos was at the same order of magnitude as in contraconceptive products currently on the market in the EU.

A rapid dose- and time-dependent impairment in embryo survival and development was observed when dechorionated zebrafish embryos were exposed to a NA/EE mixture at a fixed ratio of 500:1 for up to 48 hours. Early stage embryos were more sensitive than late stage embryos. Reduced movement of embryos were also observed within 1 hour. The NA/EE mixture at a high concentration causes a general cell death and a decrease in cell proliferation in zebrafish embryos. Zebrafish embryos exposed to the NA/EE mixture also presented misplacing, mispatterning and stunting of intersomitic vessels outgrowth and defasciculation of axons and shortening of axonal outgrowth. Exposure and uptake of NA was confirmed in zebrafish embryos exposed to the NA/EE mixture.

The authors claim that the results demonstrate that the components of Primodos (i.e. NA and EE) are potentially teratogenic. However, the available data is not considered sufficient by the CHMP to support a direct teratogenic effect of the NA/EE mixture. It is noted that some effects indicative of teratogenicity such as bent spine, pericardial oedema and yolk sac oedema were reported; however detailed individual data is missing. The study is focused on measurements of reduction in size following exposure to high (and lethal) concentrations, whereas additional, quantitated end-points related to malformation following exposure to non-lethal concentrations would have been of interest for the interpretation of teratogenicity.

Concentrations at which effect on embryonic growth and development were observed coincided with embryonic lethality (20% at the LOAEL and 50% at the subsequent concentration, which was the main focus concentration of further investigations). Induction of apoptosis and reduction of cell proliferation throughout the embryo (e.g. not localised to specific tissues) were shown at the concentration inducing 50% lethality. Concluding from this data, development related adverse effects in the zebrafish can be assumed to be related to general toxicity at this concentration. In order to accurately test zebrafish developmental toxicity, concentrations tested should not cause lethality and a maximum tolerated concentration, as defined by OECD, should preferably used, at which a maximum of 10% lethality is achieved.

As NA and EE are expected to accumulate in the yolk sack due to their high lipophilicity (Andersen *et al.,* 2003), yolk sac oedema and oedematous yolk sac extension may have been induced due to the high local concentrations of NA and/or EE in the yolk sac.

For both NA and EE, the tested concentrations in the water are much higher than the maximum exposures reached in human for products currently on the market (1 mg/day NA and 0.03 mg/day EE) (and Primodos; 10 mg NA and 0.02 mg EE). For NA at 10 mg dose in human, the exposure margins at the NOEC in was between 31-fold to 625-fold for total plasma and free plasma concentrations, respectively. At the concentration on which the manuscript bases consecutive experiments, the exposure margins for NA range between 125-fold to 2500-fold, for total plasma and free plasma concentrations, respectively.

For EE at 30 µg/dose in human, the exposure margins at the NOAEL in the manuscript range between 63-fold to 3125-fold for total plasma and free plasma concentrations, respectively. At the

concentration on which consecutive experiments are based, the exposure margins for EE range between 250-fold to 12500-fold, for total plasma and free plasma concentrations, respectively.

The true exposure margin for the zebrafish experiments probably lies somewhere in the high end between the total and free plasma calculations, due to both compound's lipophilic property and the large percentage of the total amount of NA which was detected in the zebrafish embryo. For both compounds, binding to plastic could be important for determining the exposure margin (Riedl and Altenburger, 2007). Ultimately a measurement of the compound concentration in the treatment water and the embryos at the end of the experiment would have provided information on the actual free concentrations of NA and EE in the water and exposure to the zebrafish embryos.

The authors claim that NA accumulates in zebrafish embryos over time and speculate that accumulation and build-up of high concentrations in human embryos may also occur. However, the data is limited to measurements of amount NA per embryo at three time points from 6 to 48 h exposure at 12.5 µg/mL. The concentrations range from 1 to 1.8 µg per embryo, not taking into account differences in weight or volume or general condition of the exposed embryo, with the highest concentration measured at 24 h. It is noted also that DMSO was used as a carrier in the experiments. No conclusions on bioaccumulation potential can be made from the presented data. A bioconcentration factor of 2.6 to 40.8 for norethisterone in adult fish has been reported, indicating a low potential to bioaccumulate (Nallani *et al.,* 2012). The exposure and uptake of NA dissolved in DMSO from the surrounding water in dechorionated zebrafish embryos is not comparable to the possible placental transfer of drug compound to a mammalian embryo. Any direct comparisons regarding uptake and possible accumulation of NA in the zebrafish embryo with a human embryo/fetus should not be made from these data or from other fish bioaccumulation data.

Investigations of blood vessel patterning and neural patterning

Due to the overt toxicity at the concentration in these zebrafish embryos experiments, resulting in 50% lethality accompanied by anti-proliferative and cytotoxic effects, it cannot be concluded if effects observed on blood vessel development and neural patterning in zebrafish embryos are induced by specific adverse effects on development or due to the general toxic effect of the mixture at this exposure level.

It has not been shown if a control compound, which inhibits proliferation and induces cell death, but it is not known to decrease blood vessel development and/or neural patterning provides a similar effect in zebrafish embryos at a similar general toxicity level (LD50). Such a control and a positive control (a known blood vessel development inhibitor and a neuro-developmental toxicant) are needed to be able to interpret the outcome of this experiment.

In the HUVEC experiment and the *in vitro* mouse retinal explant assay, to ensure validity of the experiment, a positive and negative control should also have been included. Data of NA/EE induced cytotoxicity at tested concentrations in these cell lines was not provided, which is information mandatory to interpret the results of these *in vitro* studies. However, adverse effects on cell proliferation (approximately 50% reduction at highest concentration tested) and reduced number of total cells in the HUVEC experiment are indicative of a cytotoxic mechanism by the NA/EE mixture. In public literature, the inhibitory concentration for viability (IC50) for NA was established at 5 µg/mL in RENT4 endometrial cells and at 7 µg/mL in foetal limb bud cells (Ostad *et al.,* 1998). Cytotoxicity of ethinylestradiol has been described in primary liver hepatocytes, with an IC50 of 44 ng/mL(Wan and O'Brien, 2013). Taken together, as the NA/EE mixture at this exposure level reduces proliferation, increases cell death, and an IC50 of 5-7 µg/mL of NA, has been described for other cell lines, the

adverse effects observed in the *in vitro* HUVEC and in mouse retinal explant experiments could be due to the cytotoxicity of the NA/EE mixture.

In conclusion, the norethisterone and ethinyl estradiol mixture induced effects on development and lethality/cytotoxicity in zebrafish and *in vitro* studies at concentrations of multiple orders of magnitude higher than free plasma exposure in humans after intake to Primodos, namely 625 to 2500-fold for norethisterone and 3125 to 12500-fold for ethinylestradiol, for the NOEC (no observed effect concentration) and concentration of detailed investigation in the manuscript, respectively.

However, the reliability of the performed studies could not be fully evaluated due to lack of crucial information. The available data is not considered sufficient to support a direct teratogenic effect of the NA/EE mixture or of the individual components.

The results of an alternative reproductive toxicity study performed *in vitro* or in non-mammalian species need to be evaluated together with all available *in vivo* non-clinical and human data, including exposure data, as part of an integrated risk assessment approach, in line with approach described in guideline ICH S5 (R3).

Indeed, Brown and colleagues (2018) concluded that further work in mammalian species is required to determine if placental embryos also are affected by synthetic sex hormones. However, already existing data was not taken into account and it can be concluded that the results of the presented study do not raise any new concerns that will warrant further studies in mammalian species.

2.4. Clinical relevance of findings

Published clinical data on the association between exposure to combined hormonal contraceptives (CHCs) during early pregnancy and birth defects.

Although the exposure with Primodos is very limited and not comparable with the daily exposure to combined hormonal contraceptives (CHCs) containing norethisterone or norethisterone used as monotherapy, reference is made to information in published literature on exposure of CHCs during early pregnancy.

CHCs are the most frequently used contraceptive method in the Western world. Today, there are about 100 million women who use a CHC around the world. Despite that CHCs being very effective when used according to the regimen, it has been estimated that about 9% of women nevertheless will get pregnant in their first year of use (Trussell, 2011), because of missed pills, possible interactions with concomitant medication, disease, or failure of the method. In such situations the woman might be unaware of the pregnancy and could inadvertently expose the foetus to exogenous hormones of the CHC.

There are no specific studies per type of combined CHC, but there are a number of studies which investigated inadvertent exposure by CHCs in general during early pregnancy and the risk of major birth defects, including a meta-analysis (Bracken, 1990). The meta-analysis, based on 12 prospective studies, did not find an association. The largest and most recently published study (Charlton *et al.,* 2016) is a very large Danish prospective cohort study in which it was investigated whether oral contraceptive use around the time of pregnancy onset is associated with an increased risk of major birth defects. All oral hormonal contraceptives were taken into consideration, i.e. combined oral contraceptives, progestogen-only contraceptives and emergency contraceptives. The data on oral contraceptive use and major birth defects were collected among 880,694 live births from Danish registries between 1997 and 2011. The main outcome measure was the number of major birth defects throughout one year follow-up (defined according to the European Surveillance of Congenital

Anomalies classification). Based on the results, the authors concluded that oral contraceptive exposure just before or during pregnancy does not appear to be associated with an increased risk of major birth defects (Charlton *et al.,* 2016). This study did not contain specific information on the active ingredients of the oral hormonal contraceptives taken into account, but as norethisterone containing CHCs are on the market since the 1970s it is likely included.

The findings in the zebrafish study are consistent with findings in mammalian non-clinical species and no novel signal indicating teratogenicity has been presented in the zebrafish study. There is no clear non-clinical evidence that a combination of NA and EE causes foetal malformations in non-reproductive tissue. The findings in the zebrafish study are not considered to give rise to any new concerns and further studies in mammalian species are not called for. Therefore there are no new clinical implications based on the findings in the presented zebrafish study.

3. Safety working party (SWP) consultation

The Safety working party (SWP) was asked by the CHMP to comment on the suitability of the zebrafish model for evaluating the potential effects of norethisterone and ethinylestradiol in human pregnancy.

In its response, the SWP mentioned that although not qualified for regulatory purposes, the zebrafish embryotoxicity test is currently used as a model to screen for compound induced adverse effects on embryonic development. Whilst it can not be solely relied upon to predict embryotoxicity, this can add useful information when assessing the totality of evidence. Therefore, the zebrafish model can be used to evaluate effects of norethisterone and ethinyl estradiol on embryonic development.

However, some uncertainties have to be kept in mind. Receptors targeted by norethisterone and ethinyl estradiol have been described, characterised and functionally analysed in the zebrafish model. Between zebrafish and human, structural differences between these receptors exist, and therefore it is unclear whether their role in physiology is comparable between zebrafish and human. Further, developmental defects observed in animal studies, including the zebrafish model, cannot be directly extrapolated to possible effects on human pregnancy. When animal models of multiple species show similar phenotypic effects, this increases concern for extrapolation to human.

Therefore, for hazard assessment of the human situation, the zebrafish model should be seen as complementary to the currently available tests mandated by ICH S5 (R2). For interpretation of possible adverse effects on human, the total body of available non-clinical and human evidence should be taken into account.

On the robustness of the study performed by Brown and colleages (2018), the SWP observed multiple issues and uncertainties in the methods and results of the manuscript.

Regarding the methods, main issues included lack of negative and positive controls, lack of cytotoxicity assessment for the *in vitro* cellular assays. Description of the method for morphological assessment of the zebrafish embryos was very limited and not performed to the level of current practice, lack of information on the procedure and timing of de-chorionation of the embryos, and a number of additional methodological issues. In addition, norethisterone and ethinyl estradiol were only tested in a mixture with a ratio of 500:1 in all experiments. Norethisterone and ethinyl estradiol were not tested separately in any of the models used. Therefore, it is not possible to provide a conclusion on separate effects of these two compounds on zebrafish development.

Regarding the results, the effect on development of the norethisterone and ethinyl estradiol mixture were mainly based on exposures inducing lethality (20%-50% lethality), inducing cell death and reducing proliferation in the zebrafish. However, based on current test protocols of the zebrafish

embryo developmental toxicity test, concentrations tested should not cause lethality over a maximum of 10%.

The norethisterone and ethinyl estradiol mixture induced effects on development and lethality/cytotoxicity in zebrafish and *in vitro* studies at concentrations of multiple orders of magnitude higher than free plasma exposure in humans after intake to Primodos, namely 625 to 2500-fold for norethisterone and 3125 to 12500-fold for ethinyl estradiol, for the NOEC (no observed effect concentration) and concentration of detailed investigation in the manuscript, respectively.

When discussing any clinical implications resulting from this study, the SWP concluded that due to the multiple limitations of the manuscript (Brown *et al.,* 2018) the results of this study do not add to the current knowledge regarding adverse events in early pregnancy in human. As such, the presented results do not have clinical implications as the study can not be relied upon due to the previously mentioned methodological limitations.

The conclusion, that current non-clinical and clinical data available does not support a signal of teratogenicity of a combination of norethisterone and ethinyl estradiol, remains valid.

The SWP made also two additional remarks.

A comprehensive review by the Expert Working Group on Hormone Pregnancy Tests of, at that time, available non-clinical literature did not show a causal association between administering norethisterone and ethinylestradiol at the doses and durations found in Primodos and the development of malformations in non-reproductive tissues of the offspring. The results of the zebrafish study do not alter this conclusion.

The SWP has also considered whether further clarification on the uncertainties and limitations of the study by Brown and colleagues (2018) highlighted in this report is needed. Due to the high exposure margins together with the profile for general toxicity, irrespective of the methodological issues, the outcome of the study is not relevant for the human situation. Therefore, further clarification by the authors was not deemed necessary.

4. Conclusions

Primodos was a medicinal product containing 0.02 mg ethinyl estradiol and 10 mg norethisterone. One tablet was to be taken each day for 2 days.

Whilst Primodos is not available on the market anymore, both components, i.e. norethisterone and ethinylestradiol, are still widely used.

The synthetic oestrogen ethinylestradiol is the oestrogen component in all combined hormonal contraceptives (CHCs) that are available on the market, except for two authorised CHCs which contain estradiol valerate. The dose of ethinylestradiol in CHCs ranges between 0.015 – 0.035 mg. Contrary to the oestrogen component, the progestogen component varies in CHCs. There are several different synthetic progestogens including norethisterone. The norethisterone dose in CHCs ranges between 0.5 and 1 mg.

Norethisterone and several other progestogens are also used as monotherapy or in combination with an oestrogen for the treatment of menstrual bleeding irregularities and endometriosis. Norethisterone is used for menstrual irregularities in a dose range of 5 -15 mg and as monotherapy for endometriosis in a dose of 10 mg/day.

The CHMP assessed the results of the study perfomed using the zebrafish embryotoxicity test (Brown *et al.,* 2018). The CHMP took into account the response of the SWP, regarding the reliability of the

zebrafish model in the preclinical seting, the robustness of the study and the potential clinical implications.

If appropriately qualified, a well-performed zebrafish embryotoxicity test may contribute to the evaluation of the teratogenic potential of a compound as part of an integrated testing strategy. A proper qualification of a Zebrafish embryotoxicity test has not yet been performed and it is premature to conclude on its suitability to predict potential teratogenic effects of norethisterone and ethinylestradiol in human pregnancy. The results of such a study still needs to be evaluated together with all available *in vivo* non-clinical and human data, including exposure data, as part of an integrated risk assessment approach.

The data evaluated as part of this procedure indicates effects on survival and development of the zebrafish embryo following direct exposure of a mixture of NA and EE in a ratio of 500:1 at multiple orders of magnitude higher than free plasma exposure in humans after intake to Primodos. However, the reliability of the performed studies could not be fully evaluated due to methodological limitations. The available data is not considered sufficient for establishing a direct teratogenic effect of the NA/EE mixture or of the individual components.

The results of the zebrafish embryotoxicity test study are consistent with the results of studies in non-human mammalian species. There is no clear and consistent non-clinical evidence that a combination of NA and EE causes foetal malformations in non-reproductive tissues. The results of the study by Brown and colleagues (2018) are not considered by the CHMP to give rise to any new concerns.

Overall due to the multiple limitations of the study described in the manuscript (Brown et al., 2018) the results of this study do not add to the current knowledge regarding adverse events in early pregnancy in human. The CHMP concluded that there are no new clinical implications based on the results of the presented zebrafish study.

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