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TITLE

Toward a better understanding of the effects of endocrine disrupting compounds on health: human-relevant case studies from sheep models.

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Highlights

- Endocrine disruption is a very complex and critical public health issue
- There is a need for human-relevant models to assess endocrine physiology and the physiology of period at risks
- The sheep is a good alternative to classical rodent models to understand fetal exposure, thyroid disruption and its consequences on brain development
- The sheep allows real-life exposure scenario to complex mixtures.

Abstract:

There are many challenges to overcome in order to properly understand both the exposure to, and effects of, endocrine disruptors (EDs). This is particularly true with respect to fetal life where ED exposures are a major issue requiring toxicokinetic studies of materno-fetal exchange and identification of pathophysiological consequences. The sheep, a monotocous large size species is very suitable for *in utero* fetal catheterization allowing a modelling approach predictive of human fetal exposure. Predicting adverse effects of EDs on human health is frequently impeded by the wide interspecies differences in the regulation of endocrine functions and their effect on biological processes. Because of its similarity to humans as regards gestational and thyroid physiologies and brain ontogeny, the sheep constitutes a highly appropriate model to move one step further on thyroid disruptor hazard assessment. As a grazing animal, sheep has also been proven to be useful in the evaluation of the consequences of chronic environmental exposure to “real-life” complex mixtures at different stages of the reproductive life cycle.

Key words: endocrine disruptors – sheep model –fetal exposure- thyroid - mixture

1. INTRODUCTION

Many *in vitro/ex vivo* tools have recently been developed and have proved to be very useful for rapidly screening and/or understanding of the mechanisms of action of endocrine disruptors (EDs). However, the complexity and the diversity of the effects of EDs underline the need for integrated *in vivo* models based on the appropriate use of species relevant to human for hazard assessment. This is a prerequisite to minimise the levels of uncertainty when extrapolating from animal findings to human. Rodent models are extensively used in toxicology, including endocrine toxicology, and are considered as the reference species for regulatory toxicology purposes. These models have many advantages in terms of both study duration and feasibility. These include reasonable extrapolation of some representative functions to the human, complete genetic and molecular characterisation and affordable costs. Many experimental studies have demonstrated that low doses of common pollutants, such as BPA (bisphenol A), can disrupt endocrine function, brain development and reproduction (Heindel et al., 2015; Le Magueresse-Battistoni et al., 2018; Caporossi and Papaleo, 2017), 2017; Mhaouty-Kodja et al., 2018; Palanza et al., 2016; Viguié et al., 2018) in rodents exposed during intrauterine life. The rodent models have, thus, been successfully used in pioneering studies establishing the proof of concept for the existence of EDs and their related adverse effects on the health of individuals and/or their progeny. However, the process of extrapolating experimental animal data to the human, requires an additional step towards maximising the human-relevance of the different models. This step must be based on an improved consideration of interspecies differences in the developmental and physiological scheme of regulation of endocrine functions. The challenges and questions paving the way toward a better understanding of ED effects are numerous and it is hardly conceivable that a single non-human species could enable the production of a precisely human-relevant picture for every physiological system and developmental stage. It would be a matter of grave concern, therefore, should the scientific community and the regulatory bodies rely solely on approaches exclusively designed in and for rodents.

The existence of windows of sensitivity to ED actions that result in potentially adverse transgenerational effects is a critical issue and a challenge for the ED research field. The hormone dysregulating properties of EDs are of great importance if exposure occurs during early developmental stages. This is when hormones are exerting permanent effects on fetal development by interacting with

the *in utero* processes of biological functional programming (Catanese et al., 2015). Thus, the prediction of fetal exposure to ED is a major issue that should not be neglected. It is noteworthy that such considerations are now being incorporated into some research funder priorities, as for example in the EU Horizon 2020 programmes around the exposome https://ec.europa.eu/info/funding-tenders/opportunities/portal/screen/opportunities/topic-details/sc1-bhc-28-2019. Fetal development proceeds from complex multilevel interactions between the mother and the fetus. The great differences in gestational physiology and, above all, endocrine function, which exists between rodents and humans have led to serious concerns about extrapolating rodent data to humans (Habert et al., 2014). Sheep have long been used as an animal model for studying fetal physiology and much of the available information on maternal-fetal pharmacokinetic has been obtained from pregnant ewe (Szeto, 1982). Together with the fact that characterising fetal exposure is a critical issue in the field of EDs, these historical data prompted us to develop an *in utero* catheterised ovine fetus model (Meschia et al., 1965) to determine bi-directional transplacental exchanges of bisphenol A (BPA) and its main metabolite BPA-glucuronide (BPA-G). It is noteworthy that the relevance of this model goes far beyond EDs since it is relevant to any contaminants for which fetal exposure is at high risk. This model enables maternal and fetal compartments to be assessed separately and allows direct fetal administration of EDs, thus by-passing maternal metabolism if required. We use this system to develop a pharmacokinetic-based modelling approach to predict the extent of human fetal tissue exposure to the active form of BPA and by comparison to BPS (Corbel et al., 2013; Gauderat et al., 2017; Grandin et al., 2019, 2018).

One major source of uncertainty when predicting the potential adverse effects of EDs on human health relies on coping with the considerable inter-species differences in the regulatory pathways and feedback loops of endocrine functions and their effect on critical biological processes. It is all the more worrying, therefore, that regulatory toxicological *in vivo* evaluations rely mainly on rodent studies. Thyroid disruption is an emblematic example of how the relevance of rodent results to humans can be debatable. We have come to the point when some of the inter-species differences in thyroid function regulation are being used as an argument to refute results obtained in rodent models as not relevant to humans.

Finally, one major challenge when investigating the toxicology of environmental contaminants concerns the identification of human-relevant exposure systems/models. This is rendered even more difficult by the fact that both human and animal populations are likely to be exposed to a multitude of contaminants at a time and at low doses. One approach is the use of sentinel species who share the human environment, as in a recent study comparing the effects of two EDs on dog and human sperm but these do not necessarily address complex mixtures (Sumner et al., 2019). One other possible key strategy to address this critical challenge is to expose the test animals to "real life mixture". There is some debate among the scientific community on the precise criteria for a "real life mixture" and the correct approaches to address this question. Exposing animals through their "natural" environment is one very interesting option that cannot be conducted in rodents. Using grazing animals that can be "naturally" exposed through their environment due to their feeding behaviour has been successfully developed in the sheep, notwithstanding the digestive and metabolic differences posed by the rumen in the ovine.

Considering the most human-relevant strategies to evaluate the health and developmental risks of EDs, our goal here is to show, using the sheep model as a proof of concept, how a non-rodent animal model can complete and, in some instances, validate classical regulatory rodent studies. We chose three well documented examples from ovine ED studies to demonstrate how using non-rodent models can be very useful to answer to some of the main scientific challenges in the ED research field. More specifically, we addressed the questions of the characterisation and/or prediction of exposure during fetal life one of the most sensitive life stages, the relevance of the model with respect to endocrine regulation through the thyroid function and the ability to set up long-term "real life" exposure studies.

2. MODELING MATERNO-FETAL EXCHANGES OF TOXICANTS

2.1. Fetal sheep to predict human fetal exposure to an endocrine disruptor from semi-physiological toxicokinetic models: application to the assessment of fetal exposure to bisphenols

Exposure to chemicals during prenatal life is a major concern for toxicologists. Fetal exposure to endocrine disruptors (EDs) during critical windows of development has repeatedly been suggested to

be involved in the onset of a variety of adverse biological effects, later in life, even when exposure occurs at low doses (Vandenberg et al., 2012).

A sound interpretation of human health implications of ED exposure, based on toxicological studies carried out in animal models, requires to be able to determine the human blood and/or urinary concentrations of biologically active compounds. These can be compared with the concentrations measured in test species that have responded adversely to exposure through the mother. Indeed, it is typically acknowledged that any adverse systemic effect of a compound is directly related to the plasma concentrations of its biologically active form/s. Such relationships more often follow a classical linear or monotonic pattern, but it should be noted that non-monotonic dose responses, which are very difficult to characterise, have been reported for many EDs. The biomonitoring data regarding the concentrations in human maternal, fetal, and neonatal fluids are of the utmost importance to provide a reliable foundation for the extrapolations of risks, from animal studies to humans. However, measuring fetal chemical concentrations is difficult in humans and very few measurements of EDs in the human fetal circulation have ever been made. The quantification of low levels of xenobiotics is hampered when the molecule of interest is ubiquitous in the environment and/or in tissue/sample collection, processing and analytical devices as is the case for BPA (Ye et al., 2013). Hence, the high values of cord blood BPA concentrations (in the ng/ml range) reported in some human biomonitoring studies (Zhang et al., 2014) should not be included in the risk assessment of BPA due to possible contaminations specifically related to exposure during delivery or to post-exposure sample contamination (Dekant and Völkel, 2008).

The glucuronidation of drugs constitutes a major pathway of elimination and glucuronide metabolites have been detected in fetal plasma following maternal drug administration, as it is the case for BPA. Hence, toxicokinetic studies have shown that BPA glucuronide (BPAG) accumulates in the plasma of fetal sheep and monkeys following maternal administration (Corbel et al., 2013; Patterson et al., 2013; Viguié et al., 2013; Vom Saal et al., 2014). Due to their lack of estrogenicity (Matthews et al., 2001; Skledar et al., 2016), the systemic exposure to bisphenol conjugated metabolites is not taken into account for risk assessment purposes. However, *in vitro* studies suggest that BPA glucuronide may exert biological activities similar to (Boucher et al., 2015) or different from those of the parent

compound (Viñas et al., 2013). This highlights the need for experimental data to elucidate the maternal-fetal toxicokinetic behaviour of bisphenols and to provide precise time-dependent measurements of the plasma concentrations of both the parent and glucuronidated metabolites. These data are essential for interpreting human biomonitoring data and for the development of toxicokinetic models to predict the extent of human fetal internal exposure, under an appropriate scheme of dietary and environmental exposures.

2.2. Choice of a relevant animal model: the sheep for studying maternal-fetal pharmacokinetics

Pregnant ewes have been used extensively for translational research and have contributed to very important advances in prenatal human medicine (Morrison et al., 2018). Due to the large size of these animals, catheters can be surgically inserted and maintained in both the maternal and fetal vasculature. Hence the chronically catheterised fetal sheep provides a unique model that permits the serial sampling of maternal and fetal blood, as well as other fluids, after maternal or fetal administration (Rurak et al., 1991). This animal model has already been applied to evaluate the disposition of several drugs during the prenatal period (Kumar et al., 1997; Ngamprasertwong et al., 2016). Furthermore, considerable insight regarding nutrient profiles has been gained from investigations of nutrient exchange in pregnant sheep (Barry and Anthony, 2008) highlighting important physiological similarities between the sheep and human placental functions despite structural differences. Indeed, although the sheep synepitheliochorial placenta differs histologically from the human hemochorial placenta, the microarchitecture of the ovine cotyledon is structurally analogous to that of the human placenta (Barry and Anthony, 2008; Leiser et al., 1997; Morrison et al., 2018).

Drug concentrations in fetal plasma result from a complex interplay between bidirectional placental clearances and fetal metabolism. For many drugs, in particular the most lipophilic and the low molecular ionized ones, the most important mechanism of transfer across the placenta is the passive diffusion. In this case, the extent of drug transport is dependent upon not only the physicochemical properties of the drugs but also maternal and fetal blood flows (Rurak et al., 1991). Relative to the fetal body weight, the blood flow in the umbilical vein and artery is of the same order of magnitude in sheep

(12 L/h.kg; Faber and Green, 1972; Rudolph, 1985) as in humans (7 L/kg.h, (Clavero et al., 1973; Haugen et al., 2004), indicating that the flow-limited transfer of drugs across the placenta in sheep and humans should be comparable. Accordingly, uteroplacental blood flow at the end of pregnancy is quite well preserved between rabbit, rodents, macaque, sheep and human, when normalized by the placenta weight (Mourier et al., 2017). By contrast, there are important interspecies differences in active transport of molecules (Walker et al., 2017).

It has been shown that fetal sheep, compared to adults, possess low levels of some UDP-glucuronosyltransferases (UGT) genes and thus likely lower activity (Pretheeban et al., 2011), similar to humans (Burchell et al., 1989), suggesting a lower capacity of the metabolic pathways in which these enzymes are involved in fetuses. In agreement with this observation, the intrinsic clearance of BPA glucuronidation in near-term fetal sheep (120–135 days of gestation) is about 2-fold lower than that of adults, the latter being of the same order as in adult humans (Corbel et al., 2015). Since a significant fraction of venous umbilical flow, about 75% in humans and 50% in sheep, passes through the fetal liver before reaching the systemic circulation, some pre-systemic conjugation can occur between the maternal and fetal circulations in both the sheep and human fetus (Edelstone et al., 1978; Haugen et al., 2004).

2.3. Toxicokinetic modelling of fetal exposure to bisphenol A

Toxicokinetic studies of BPA have been performed using the chronically catheterised fetal sheep model to evaluate the time-course of BPA and BPAG concentrations in maternal and fetal plasma following maternal and fetal BPA or BPAG intravenous dosing (Corbel et al., 2014; Gauderat et al., 2016). This approach was applied to determine the relative contributions of the different pathways controlling fetal exposure to BPA. The parameters estimated by the model are summarized in fig. 1 right-hand panel. At the end of pregnancy, about 6% of the maternal BPA dose enters the fetal circulation, *i.e.* a dose relative to body weight equivalent to the maternal dose. The closeness of this value with that estimated at 3.1% in human, using and *ex-vivo* model of perfused human placental cotyledon (Corbel et al., 2014), emphasizes the relevance of the sheep model for studying placental drug transfer. Most of the BPA entering the fetal circulation was rapidly eliminated, mainly through

the direct clearance of BPA from the fetus to the mother. This represented approximately 74% of the fetal clearance of BPA. The remaining part of fetal BPA clearance (26%) was accounted for by the fetal metabolism of BPA into BPAG. Although not the predominant mechanisms of BPA elimination, the fetal conjugation clearance of BPA was higher than in the adult, despite the lower fetal expression of the UGTs involved in fetal BPA glucuronidation (Pretheeban et al., 2011). This high fetal conjugation clearance of BPA was responsible for a significant first-pass conjugation of BPA transferred from the mother to the fetus. The limited fetal to maternal transfer accounted for about only 17% of the total BPAG output. Therefore, the elimination of BPAG trapped in the fetal compartment mainly involved BPAG hydrolysis (83%).

A non-linear mixed effect model (NLMEM) was used to simultaneously analyse these toxicokinetic data and build a semi-physiologically-based toxicokinetic model of feto-maternal BPA exchange (Gauderat et al., 2017). The semi-physiologically-based toxicokinetic model of feto-maternal BPA toxicokinetic developed in sheep was then humanised (Gauderat et al., 2016), using toxicokinetic data obtained in adult humans via ingestion of a BPA (d6-BPA) labelled cookie. This enabled the prediction of BPA and BPAG concentration-time profiles in human maternal and fetal plasma. After a pattern of repeated maternal exposure to BPA that mimics dietary exposure to the average dose estimated by EFSA ((EFSA, 2015), 11.05 nmol/d for 70 kg body weight), the toxicokinetic model predicted human plasma concentrations of non-conjugated BPA fluctuating between 14 and 140 pg/L in the adult and between 11 and 23 pg/L in the fetus, *i.e.* well below some of the very high concentrations (several hundred ng/L) of unconjugated BPA in cord blood reported in some human biomonitoring studies (Gerona et al., 2013; Veiga-Lopez et al., 2015). The maternal plasma BPAG concentrations predicted by the toxicokinetic model fluctuated considerably (between 1.6 and 40 ng/L) depending on the delay between measurements and ingestion of BPA, while the mean BPAG-predicted concentrations in the plasma of the human fetus were relatively stable and in the order of 40 ng/L. The consistency of these predictions with measurements of BPAG (median values of 120 and 960 ng/L, (Gerona et al., 2013; Veiga-Lopez et al., 2015) or total BPA (mean value of 46 ng/L; (Yamamoto et al., 2016) in human cord blood again emphasises the relevance of the pregnant sheep model to predict human feto-maternal toxicokinetics of BPA.

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239 **2.4. Toxicokinetics of bisphenol S**

240 Owing to serious concerns regarding BPA the adverse effects of BPA on human health, it has been
241 replaced by structural analogues, mainly bisphenol S (BPS, (Liao et al., 2012). The high prevalence of
242 BPS in urine from a cohort of US adults (Ye et al., 2015), and its detection in human cord blood
243 (Kolatorova et al., 2018), gives rise to the question of the level of risk for human health associated
244 with prenatal exposure to this BPA analogue. Indeed, *in vitro* studies have shown that BPS binds the
245 estrogen receptors (ER) α and β (Molina-Molina et al., 2013) and acts as an ER agonist with a weaker
246 or equivalent potency to that of BPA (Kojima et al., 2019; Kuruto-Niwa et al., 2005; Rosenmai et al.,
247 2014). Although limited in number, *in vivo* studies have shown that prenatal exposure of mice to BPS
248 alters mammary gland development (Tucker et al., 2018). There is currently a critical gap in
249 knowledge about the extent to which the human, especially the fetus, is internally exposed to emerging
250 bisphenols. The only available toxicokinetic study of BPS performed in adult humans (Oh et al., 2018)
251 suggests that the toxicokinetic behaviour of BPS may differ from that of BPA and highlights the need
252 for animal studies of BPS toxicokinetic to identify the underlying mechanisms of gestational exposure
253 to BPS.

254 The main metabolite of BPS formed *in vivo* is BPS glucuronide (BPSG, Le Fol et al., 2015).
255 Toxicokinetic studies of BPS and its major metabolite, were carried out using a chronically
256 catheterised fetal sheep model to evaluate the time-course of BPS and BPSG concentrations in
257 maternal and fetal plasma following maternal and fetal BPS or BPSG intravenous dosing (Grandin et
258 al., 2018). It was striking that the fraction of the maternal BPS dose transferred from mother to fetus
259 (0.40%) was about ten times lower than that of BPA (Gauderat et al., 2017; fig.1). A limited placental
260 passage of BPS has also been revealed using an *ex-vivo* model of the perfused human placental
261 cotyledon model (Grandin et al., 2019). In this model, the materno-fetal clearance index (0.0852) was
262 about 10-fold lower than that of BPA. In sheep, only 26% of the BPS dose entering the fetal blood
263 against 74% for BPA (fig.1) were rapidly eliminated through its transfer into maternal blood. This
264 result is in agreement with the higher efficiency of fetal to maternal transfer as compared to its
265 maternal to fetal counterpart evidenced in the perfused human placenta (Grandin et al., 2019). About

half of the remaining BPS (46%) was metabolised by the fetus into BPSG. The elimination of this BPSG from the fetal compartment required its hydrolysis, like that of most of the BPAG, due to a limited placental transfer. Our compartmental toxicokinetic model predicted that, despite a lower materno-fetal passage of BPS compared to BPA, the higher persistency of BPS in the fetal compartment leads to expected BPS concentrations in fetal plasma of the same order than that of BPA (Grandin et al., 2018).

3. INTERSPECIES DIFFERENCES IN ENDOCRINE REGULATION: THE EXAMPLE OF THE THYROID FUNCTION

Although the basic mechanisms of endocrine regulation, in particular feedback mechanisms, are well preserved among vertebrates, there are interspecies differences in the pathways mediating those mechanisms. This raises concern about the relevance of animal models for human endocrinology and our capacity to extrapolate results from animal to human when dealing with EDs. The sheep is a human-relevant model to elucidate the regulation of several endocrine systems, including the somatotropic, corticotropic, reproductive and thyroid systems in terms of both physiological and exposed conditions (Dutour et al., 1997; Evans et al., 2016; Karsch and Battaglia, 2002; Puttabyatappa et al., 2019). Unfortunately, in sheep as in other species, the ED research field is, to a large part, limited to the reproductive, metabolic and thyroid functions. The interest in thyroid function is justified by the relatively high incidence of thyroid dysfunctions in humans. Overall in Europe, there are around 200 new cases of thyroid disease/100,000/year. This is highest for hypothyroidism at 127 new cases/100,000/year (for review see Garmendia Madariaga et al., 2014). Furthermore, epidemiological and meta-analysis studies indicate a high incidence of undiagnosed thyroid function abnormalities (Garmendia Madariaga et al., 2014), including in pregnant women (Andersen, 2019).

The fact that neural development and associated behavioural and cognitive capacities can be altered in response to even mild thyroid disruption has reinforced the increasing ED research focus on thyroid disruption. A prevailing hypothesis is that exposure to chemical contaminants targeting thyroid function, during critical windows of sensitivity in terms of thyroid and/or neural development, might play a substantial role in the development of such disorders (Zoeller et al., 2002). The regulatory evaluation of

developmental toxicity of chemicals is based on specific guidelines. In mammals, despite ongoing debate on the relevance of rodent models toward human thyroid physiology, the only *in vivo* proposed OECD guidelines for thyroid are rodents screening assays (OECD series on testing & assessment, 2012, n°150, 524). Thyroid investigations in those tests are limited and are not specifically designed to identify thyroid disruption. They are included in male and female pubertal assays, described by US EPA (US EPA OPPTS 890 1450-1500), and other repeated doses toxicity assays (OECD TG 407, 416, 443). These are based on amphibian studies or, in mammals, on thyroid hormones (TH) and/or Thyroid Stimulating Hormone (TSH) measurement in rodents at a given time point, usually at termination with, in the best case, histological evaluation of the thyroid gland. The OECD guidelines for 28 and 90 day repeated adult rodent exposure studies stipulate: "although in the international evaluation of endocrine related endpoint a clear advantage for the determination of TH or TSH could not be demonstrated, it may be helpful to retain to measure T3, T4 and TSH if there is an indication for an effect on the pituitary-thyroid axis. Definitive identification of thyroid-active chemicals is more reliable by histopathology analysis" https://www.oecd-ilibrary.org/environment/test-no-407-repeated-dose-28-day-oral-toxicity-study-in-rodents_9789264070684-en.

3.1. TH metabolisms and specific binding proteins

All levels of regulation of thyroid function are potential targets for environmental contaminants (for review see: (Howdeshell, 2002; Zoeller, 2010). However, one of the most frequently reported mechanisms of action concerns the hepatic metabolism of thyroid hormones (TH, fig. 2). This is essentially based on extensive experimental data in rodents. In animals as in humans, hepatic catabolism of TH is mainly via glucuronidation catalysed by hepatic microsomal enzymes from the UDP-glucuronyltransferase (UGT) and or cytoplasmic sulphotransferase (SULT) families. TH can also be metabolised by deiodinase enzymes, leading to either inactivation as for example the transformation of T3 to the inactive metabolite rT3 or bioactivation of T4 into T3. About 80% of circulating T3 comes from hepatic deiodination of T4. Both conjugations and deiodination pathways can be targets of EDs but conjugation pathways are the most frequently investigated.

Among factors contributing to the inter-specific differences in hepatic TH metabolism, plasma transport proteins of high specificity and affinity for TH namely thyroxin binding globulin (TBG) and transthyretin (TTR), are at the heart of the controversy over the relevance of rodent models to humans. These proteins are common targets of several EDs. For example, data from biosensor assays have indicated the potent binding by hydroxylated metabolites of the polybrominated diphenyl ethers (PBDEs), BDE47, 49 and 99 to human TBG (Marchesini et al., 2008). The same biosensor assay confirmed the binding capacity of TTR for hydroxylated metabolites of PCBs and PBDEs, halogenated BPA and genistein. The expression profiles of these proteins differ markedly between the adult rodent and humans. The main difference lies in the level of expression of TBG, the protein with the highest affinity and specificity for thyroxine. It is indeed strongly expressed in humans, although subject to some variations in expression according to the physiological status. TBG is only expressed at very low levels in the adult rat, but at higher levels in very young animals or aged individuals (Larsson et al., 1985; Mendel et al., 1987; Savu et al., 1991). The differences in half-life and/or clearance of thyroxine are frequently attributed to the expression profile of TBG, which is thought to be involved in decreasing the accessibility of circulating TH to hepatocytes, thereby limiting the catabolism of these hormones. These differences in the pattern of plasma TH binding proteins often lead to the assumption that rats are more sensitive than humans to thyroid disruption proceeding from an increased hepatic catabolism of TH and are used as an argument to refute the results obtained in rodents. For example, the effects of both the insecticide fipronil (European Food Safety Authority (European Food Safety Authority (EFSA), 2006) and the no-phthalate alternative plasticizer 1,2-Cyclohexane Dicarboxylic Acid, Di-Isononyl Ester (DINCH, (Bhat et al., 2014) on thyroid function were considered as not relevant to human during the course of their regulatory evaluation based on the interspecies difference in TBG expression. Interestingly, the profile of expression and pharmacodynamics properties of these proteins are very similar between human and sheep (fig. 3).

It is noteworthy, that, at the level of the EU the revised guidance for the identification of EDs states that, “so long as not demonstrated as totally irrelevant to human, all results in rodents showing an effect of a xenobiotic on TH metabolism should be considered as relevant to humans” (European Chemical Agency (ECHA) and European Food Safety Authority (EFSA) with the technical support of the Joint

Research Centre (JRC) et al., 2018). It remains true anyway that, to date, it is unclear to what extent inducible UGT/SULT expression and activity can mediate disturbances of TH catabolism in humans. The ECHA recommendation for a better consideration of rodent data for the evaluation of potential thyroid disruptors can thus be viewed like a precautionary measure in face of the uncertainties related to the human-relevance of animal models for this particular mechanism.

The induction of enzyme pathways involved in thyroid metabolism is often mediated through the activation of Xenobiotic Nuclear receptors (XNRs; (Kretschmer and Baldwin, 2005) and is not strictly specific of thyroid hormones. Most of these receptors exhibit wide interspecies differences in their pharmacological properties (Gibson et al., 2002; Jones et al., 2000). It is therefore very difficult to predict the hepatic inductive properties of xenobiotics in humans on the basis of data collected in rodents. Our work on thyroid disruption in response to fipronil provides a good example of how interspecies differences render dealing with the issue of the relevance of animal models toward human so difficult when looking at TH hepatic catabolism. The effect of the insecticide fipronil and/or its much more persistent metabolite, fipronil sulphone, on thyroid function has been well established in the rat. Both fipronil and its main metabolite fipronil sulphone treatments increase T4 clearance jointly to liver UGT activity in the rat (Leghait et al., 2009; Roques et al., 2012). In the sheep, a species expressing a very similar TBG to that of humans, the effect of fipronil exposure on thyroid homeostasis is much more limited (Leghait et al., 2010). However, it cannot be assumed at this point that these differences are solely related to different patterns of TBG expression. Pertinently, the rate of metabolic conversion of fipronil into its bioactive persistent metabolite,fipronil sulphone, differs markedly between the two species (Leghait et al., 2010) indicating differential effects on the induction of the hepatic cytochrome enzymes involved in fipronil metabolism. Fipronil sulphone is much more persistent than fipronil itself and at least as active as fipronil to increase TH hepatic catabolism. In the rat, fipronil treatment led to the upregulation of several genes involved in the metabolism of xenobiotics and or TH, including the cytochrome P450 Cyp2b1, Cyp2b2 and Cyp3a1, the carboxylesterases Ces2 and Ces6, the phase II enzymes Ugt1a1, Sult1b1 and Gsta2, and the membrane transporters Abcc2, Abcc3, Abcg5, Abcg8, Slco1a1 and Slco1a4. Our data in CAR- or PXR-invalidated mouse indicate that PXR and CAR are mediating, at least in part, the fipronil-induced upregulation of the expression of enzyme/transporter

genes (Roques et al., 2013) which, in rats, may contribute to increase thyroid hormone clearance. Given the wide interspecies differences in XNRs pharmacology, it is impossible at this point to determine if the difference in response to fipronil between the two species, in terms of thyroid disruption, is due to differential expression of TBG or to differences in XNR receptor affinities for fipronil or its metabolite or both. Although the sheep can be considered much more relevant to human than rodent from the standpoint of specific TH binding proteins, the relevance of this model to human in terms of the effect of fipronil on thyroid homeostasis is not currently certain.

3.2. Thyroid-mediated alterations of brain development

While the mode of action of EDs on thyroid function is much debated as regards hepatic initiating events, the evaluation of the adverse effects of subtle thyroid disruption is a real matter of complexity. One of the most, if not the most, challenging and worrying adverse effect of thyroid disruptors is the potential alteration of the development of the central nervous system (CNS) and resulting behavioural and cognitive deficiencies even with moderate thyroid disruption as with isolated hypothyroxinemia (Bellanger et al., 2015; Ghassabian et al., 2014; Gilbert et al., 2012a; Howdeshell, 2002). The proof of concept for linking subtle perinatal thyroid disruption, alterations of the CNS and related-cognitive deficiencies has been efficiently established in rodents, but the process still needs to be taken one step further, with integrated complementary models more relevant than rodents as regards human physiology. The period of gestation is a window of high vulnerability to perturbed thyroid function and, consequently, for developing tissues and functions which are under the control of TH. During this period, the mothers have to face increased TH requirements due to the fact that their own metabolic rate is increased to maintain a normal gestation. In addition, the needs of the fetus, particularly at early developmental stages when the fetal thyroid system is not yet functional, adds to this burden. Thus, pregnancy involves profound changes in almost all aspects of thyroid regulation. For example, the increase in TH production rate to satisfy gestational demand is estimated at about 50% of the basal rate. The maintenance of normal thyroid function is strictly–dependent upon iodine supply and/or turnover. During gestation, urinary elimination of iodine is increased because of a higher glomerular filtration rate, which itself constitutes another susceptibility factor of thyroid function. In both human

and sheep, total serum T4 and T3 concentrations rise to levels higher than those of non-pregnant individuals. BPA is a good illustration on how a contaminant that does not seem to have any effect on the thyroid function of adult non-pregnant animals might affect this function in pregnant animals and/or their offspring. There are indeed data in both rodents and sheep showing that BPA can alter the thyroid function of pregnant animals and/or their offspring (Guignard et al., 2017; Silva et al., 2019; Viguié et al., 2013; Xu et al., 2019; Zoeller et al., 2005).

The dependency of the fetus on TH maternal supply is particularly important at the early stages of the development (fig. 4). The fetal/ neonates thyroid function develops earlier in human and sheep than in rodents. Fetal thyroid function starts after the first third of the pregnancy in human as well as in sheep (fig. 4) while it starts much later, in the last third of pregnancy in rodents (Fisher, 1991; Fisher et al., 1976; Howdeshell, 2002; Polk et al., 1991). Consequently, the window of sensitivity to thyroid disruption might differ between humans and rodents. The sheep might thus represent a better alternative than rodents when investigating the window of sensitivity during which an effect of thyroid disruption on CNS development will be established.

In addition, the mode of exposure to contaminants differs between pre and post-natal life. During prenatal life, the exposure occurs from the mother through the placental barrier that in some instances protects the fetus. During post-natal life, the exposure will occur via oral route through lactation. Thus, it is almost impossible to reproduce in rodents the same scheme of exposure to contaminants at the final stage of the thyroid maturation as in humans. In addition to the effects of contaminants on the maternal thyroid function, there is evidence that domestic and/or environmental contaminant can disrupt the development of fetal thyroid. Maternal smoking and high maternal BMI are associated with disturbed fetal thyroid gland development and endocrine function in a sex-specific manner during the second trimester of pregnancy in humans (Filis et al., 2018). Similarly, peri-conceptional maternal exposure to sewage sludge chemicals impedes fetal thyroid gland development in the sheep (Hombach-Klonisch et al., 2013). BPA has also been shown to be a potential disruptor of maternal and/or fetal thyroid function following chronic high (5 mg/kg/day) or low (5 µg/kg/day) dose administration from the first to the last month of gestation in sheep (Guignard et al., 2017; Viguié et al., 2013). Interestingly, some, although not all, epidemiological dat from large human cohorts also

show a correlation between gestational BPA exposure and some degrees of thyroid disruption in newborns (Aung et al., 2017; Chevrier et al., 2013; Romano et al., 2015).

The role of TH in brain development and homeostasis is well documented in animal experimental models (Howdeshell, 2002) including for subtle thyroid disruption. In the human, although the effect of moderate thyroid disruption on behaviour and cognitive function development is still debated, some epidemiological studies suggest a potential link between maternal hypothyroxinemia (decreased free-T4 concentration with or without modifications of TSH) during pregnancy and increased risk of autism, impaired psychomotor development, Attention Deficit Hyperactivity Disorder, delay in language development and lower mental scores (for review: (Henrichs et al., 2013). The overall costs of such alterations in relation to ED exposures have been estimated at approximately 150 billions € /year in the EU (Trasande et al., 2015). In the mouse, a 36% decrease in maternal T4 concentrations without any modification of circulating T3 from gestational day (GD) 10 to GD13 is associated with a cellular and molecular environment in the cortex that is unfavourable to neuronal migration (Cuevas et al., 2005). In the rat, different models of chemically-induced moderate thyroid disruption during pregnancy (e.g. iodine deficiency, perchlorate exposure, propylthiouracil) consistently show that these manipulations are associated with functional modifications within the hippocampus with an alteration of synaptic functions that can last until the adult stage even after a full recovery of the thyroid function (Gilbert et al., 2017, 2012b; Gilbert and Sui, 2006). Subcortical band heterotopia (SBH) represent a neuronal migration error in humans. In rats, even minor (<15%) reductions in maternal serum thyroxine is associated with increased volume and presence of SBH in the corpus callosum of offspring that persists in adulthood (Gilbert et al., 2014). In rodent models, as in humans, this abnormality in brain development is associated with serious adverse neurological and/or behavioural effects. The whole process of SBH development starts in the periventricular area *i.e* in close vicinity to major sources of TH supply to the brain, the cerebrospinal fluid and vasculature.

Taken together, there is considerable rodent model evidence for the adverse effects of ED-induced mild thyroid disruption in CNS development and potential mechanisms of action ((Gilbert and Lasley, 2013; Mughal et al., 2018; Remaud et al., 2014; Zoeller et al., 2002). However, the interspecies temporal differences in the development of thyroid function, and of the CNS itself, emphasizes the

need for other animal models more relevant to human. This is especially important for the determination of the critical windows of action of EDs in the brain, neurocognitive and behavioural functions. From this standpoint, the sheep is an excellent model (Back and Hohimer, 2016), meeting several critical criteria: i) being a reference model for physiology of gestation and development (Morrison et al., 2018), ii) having similar regulation of thyroid function to that of human , iii) exhibiting time-lines for the synchronised development of the thyroid and CNS that are similar to human (fig. 4), iv) possessing considerable homology with human brain anatomy (gyrencephalic species (Vink, 2018) and maturation (a marked difference with lissencephalic species like the mouse). This makes the ovine model very valuable for the study of neurodevelopmental diseases and/or alteration in the pattern of brain development.

Overall, it is clear that regulatory studies of thyroid disruption are limited due to both the use of reference species which are controversial in terms of their relevance to human and the use of terminal endpoints and periods of observation that might only partially reflect the thyroid status of the animals and/or developmental exposure. There are very few recommendations for using other models than rodents and therefore very few data for a proper evaluation of interspecies differences, that could serve as a base for a sound extrapolation of experimental animal data to human.

4. A HERBIVORE AT THE INTERFACE BETWEEN ENVIRONMENTAL POLLUTANT AND PHYSIOLOGICAL EFFECT: A MODEL FIT FOR REAL LIFE SCENARIO EXPOSURE

The banning of disposal of human sewage into the seas and oceans by the EU in 1997 hastened the development of alternatives for the repurposing and processing of this waste material. Human sewage has long been used as a fertiliser (called “night soil”) and this trend has continued with the employment of processed, dried, human sewage as a fertiliser. Subsequently, agricultural usage laws and guidelines have been imposed. Sewage sludge is, of course, an exquisite read-out of combined man-made and environmentally distributed pollution, including a rich cocktail of EDs. The Aberdeen sewage sludge model, using outbred sheep, was established by Dr Stewart M. Rhind (deceased) at the James Hutton Institute (previously the Macaulay Institute) and has been previously reviewed (Evans et

al., 2014; Fowler et al., 2012; Rhind et al., 2010a) These reviews present considerable support for the use of sheep as a sentinel species for environmental ED effects throughout the life-course, with the added issue that, of course, sheep meat enters the human food chain. This issue has attracted considerable attention from both farming organisations and those opposed to the use of sewage sludge fertiliser.

Here we present an update that has resulted from some 20 years of research with extensive support from a range of organisations including the European Commission via the REEF project. Three main study designs have been employed in terms of the exposure paradigms via different fertilisers applied to pastures and these are summarised in fig. 5. In all three approaches fetal and maternal tissues have been harvested, typically at 80, 110 or 140 days of gestation and offspring tissues in early adulthood at around 18 months. The simplest model has been the *Always* exposure profile where mother, gestation and offspring remain exposed to a single condition. These are Control fertilised, using inorganic fertiliser balanced for nitrogen content and Sewage Sludge fertilised, using processed, dried, human sewage sludge according to agricultural guidelines at the time. The next model was the *Cross-over* exposure profile. In this model the ewes were either moved to the opposing fertiliser regime (or not, to provide additional controls) and they and their offspring then remained on that pasture type until tissue harvesting at 110 days and 18 months post-natal. The model used in the REEF project was the *Windows* exposure profile. In this model the main study controls were animals reflecting both treatment conditions of the *Always* profile, matched with ewes exposed in early (0-80 days), mid (30-110 days) and late (60-140 days) gestational windows and tissues harvested at 80 and 140 days. This design aimed to determine whether there were specific developmental windows of fetal sensitivity to EDs in sewage sludge.

The results from these three study designs are summarised in fig. 6 and demonstrated the wide range of adverse consequences from pre- and post- natal exposure to low dose, complex, mixtures of EDs in sheep.

4.1. Environmental and sheep tissue consequences of sewage sludge exposure

Unsurprisingly, sewage sludge pellets themselves contain considerably higher concentrations of 33 EDs (including DEHP, PCBs, PBDEs, PAHs) measured and reported across 3 studies (Lind et al., 2009a; Rhind et al., 2010b). The review from Evans (Evans et al., 2014) extensively assessed tissue and soil ED levels following sewage sludge application.

EDs that were detectable in soil after sewage sludge application included phthalates, alkyphenols, PAHs, PCBs, PBDEs and DEHPs. However, accumulation of EDs in soil from pastures treated with sewage sludge fertiliser was highly variable across multiple studies, temporally and geographically changing with considerable differences in accumulation of specific EDs (Rhind et al., 2002; Zhang et al., 2014) and frequently at levels that in animal studies would be considered as No Observed Adverse Effect levels (NOAEL). For any single study, therefore, accurately predicting ED load taken up by grazing animals after sewage sludge application or extrapolating more widely, is difficult. Multiple applications of sewage sludge lead to increased concentrations of chemicals and the number of congeners (PBDEs) measurable (Rhind et al., 2013) but the magnitude of the increase is lower than expected from the total sewage sludge quantities used on the pastures. An obvious explanation is that degradation or flushing of chemicals from sewage sludge-treated pasture will reduce animal exposure levels. Furthermore, since these EDs are in the environment from other sources, control pastures also contained detectable levels of many of them, although usually at lower levels than seen in the sewage sludge fertilised pastures. Relevantly for BPA, while levels in soils were initially similar between control and sewage sludge pastures, the accumulation of this compound over 13 years of application was considerable, rising from 3 to 63 ng BPA/g of soil (Zhang et al., 2015). Furthermore, there was significant uptake by crops with up to 68 ng BPA/g of leafy vegetables.

Tissue concentrations of EDs also displayed considerable variability. These ED concentrations can be influenced by the metabolic status of the animal and environmental contribution to routes of exposures, including ingestion and inhalation. The release of many lipophilic EDs from adipose stores when energy demands increase during gestation, means ED exposures are likely to be quite different between a dam and the fetus. Of the EDs of interest in the sewage sludge studies, tissue levels of PCBs, PBDEs, PAHs, alkyl phenols and phthalates are variously measurable in tissue and/or milk samples, collected from mothers, fetuses and lambs maintained on both the control and biosolids

treated pastures (Rhind et al., 2005, 2007, 2009, 2010b, 2011a) with up to 30% variation in background tissue ED accumulation across Scotland (Rhind et al., 2011b) Overall, maternal and fetal tissue levels in kidney fat, muscle and liver were elevated, even if relatively modestly and variably following sewage sludge exposure in these studies. Furthermore, while many EDs are lipophilic, even during lactation, lamb ED levels were not markedly elevated compared to the dams (Rhind et al., 2007). Different EDs also showed considerable variability between mothers and offspring and between studies. Tissue concentrations in 6 month old lambs on either treated or control pastures, showed a broadly similar pattern of tissue EDC concentrations to those seen in the adult ewes.

Overall, fetal EDC concentrations and thus EDC exposure may be greater in early gestation as a result of lower detoxification capacity of the fetal liver (Rhind et al., 2010b). In studies where exposures of the ewes at conception or during gestation were altered (*Cross-over* and *Windows* studies) different outcomes were observed. In the former, there were almost no differences between control and cross-over animal tissue ED burdens (Rhind et al., 2010b). In the *Windows* study (Lea et al., 2016), soil PAH, DEHP and PCB levels were increased by sewage sludge exposure. In dams 10 of the 15 EDs that showed significant association with sewage sludge exposure were elevated, especially in the mid and late exposure windows although changes in fetal ED burdens were highly variable. Finally, we recently showed a sex difference in 18 month old sewage sludge exposed offspring, with higher liver PAHs and lower PCBs in males than females. Furthermore, PAHs were elevated and PCBs lowered in sewage sludge-exposed females compared to controls (Filis et al., 2019).

4.2. Gonad and endocrine development

While most of our reproductive studies focused more on the female, *Always* exposed 110 days of gestation male fetuses exhibited a wide range of significant developmental defects, including reduced circulating inhibin A, testosterone (nearly halved), Leydig and Sertoli cell numbers, body and testis weights (Paul et al., 2005). A programme of analysis of the fetal ovary spanned *Always*, *Cross-over* and *Windows* studies. There was some variability between the studies, for instance in the *Always* (Fowler et al., 2008) and *Windows* (Lea et al., 2016) studies fetal ovarian weight was not altered while sewage sludge exposed fetal ovaries were heavier in the *Cross-over* study (Bellingham et al., 2013) in

fetuses whose mothers remained on sewage sludge-fertilised pastures but not in those whose mothers switched pasture type at conception. Fetal circulating prolactin and estradiol levels were reduced and FSH unaffected in *Always* study exposed fetuses while inhibin A and FSH was increased and estradiol decreased in animals constantly exposed to sewage sludge in the *Cross-over* study. In the *Windows* study, clear signs of some virilisation were seen, with reduced uterine weight and increased anogenital distance in the late window of fetal sewage sludge exposure in particular, even though fetal testosterone was unaltered in this group.

In terms of ovarian morphology, histological analysis showed that oocyte density was reduced in fetuses exposed to sewage sludge pasture (*Always* study, Fowler et al., 2008) with a shifted balance between the pro- (BCL2) and anti- (BAX) apoptotic ovarian markers. In the *Cross-over* study (Bellingham et al., 2013) in contrast, the only observed effect on follicle numbers was a reduction in the number of type 1a follicles in the fetuses whose mothers had been moved to sewage sludge pastures at mating. This was despite alterations in ovarian expression of a number of proteins/genes associated with follicle recruitment, activation, apoptosis and health. Subsequently, in the *Windows* study (Lea et al., 2016) there were several changes in numbers of different follicle classes, most notably a decrease in healthy and increase in atretic type 1a follicles in all exposed groups and indications of delayed follicle development ovaries from fetuses in the late exposure window. These changes were associated with a large number of proteome and transcriptome changes in the mid and late sewage sludge-exposed ovaries in particular. From these data network analyses predicted methylation associated changes were occurring in these 2 groups.

4.3. Brain, pituitary and thyroid development and function

Expression of GnRH and its receptor (Bellingham et al., 2010) and afferent systems to the GnRH neuronal network including Kisspeptin (Kiss-1)/GPR54, estradiol receptor alpha (ERα) (Bellingham et al., 2009), galanin and its receptors (Bellingham et al., 2010, 2009) within the hypothalamus and LHβ, Kisspeptin and ERα in the pituitary gland were affected in the sewage sludge-exposed fetuses, unlike their mothers (*Always* exposure model). GnRH and GnRH receptor mRNA concentrations were

reduced in the hypothalamus but unaffected in the pituitary glands of fetuses exposed to sewage sludge (Bellingham et al., 2010). Important steroid sensitive afferents to the GnRH neurosecretory system, galanin and kisspeptin were disrupted in fetuses exposed to sewage sludge: expression of *KISS1* transcript and the three galanin receptor subtypes (1, 2 and 3) in both the hypothalamus and pituitary glands were reduced following maternal sewage sludge exposure. Estradiol receptor transcript expression within the hypothalamus and pituitary gland were not affected by sewage sludge exposure in either the mothers or their 110 day old fetuses. However, fetal pituitary ERα protein expression was reduced in sewage sludge-exposed fetuses. A similar range of endpoints were assessed in further study using the *Cross-over* study model (Bellingham et al., 2016). Fetal plasma FSH, estradiol and inhibin A were affected by sewage sludge exposure while LH and estradiol, but not testosterone, were affected in males. The direction of disruption varied according to exposure type: constant or cross-over, with sex-specific consequences. Overall, fetuses from mothers whose exposure type changed at mating showed more extensively disrupted hypothalamic and pituitary expression patterns of neuroendocrine regulators, such as the GnRH and Kisspeptin systems, than fetuses constantly exposed to sewage sludge. Male fetuses were more affected than females and exhibited more female-like patterns of neuroendocrine gene expression. Finally, 5 month old lambs were assessed using behavioural tests indicative of emotional reactivity and exploratory behaviour. Sewage sludge exposure *in utero* and post-natally was associated with a decrease in emotional reactivity and decreased activity levels. Furthermore, the exploratory behaviour of females was unaltered but that of males increased to match female levels (Erhard and Rhind, 2004). This shows that early exposure to low levels of a cocktail of pollutants, alters brain development and dimorphism that could have long-term detrimental effects on reproductive behaviour.

The fetal thyroid gland was also investigated in the *Cross-over* study (Hombach-Klonisch et al., 2013). Fetal thyroid gland weight was only affected in the group moved from sewage sludge to control pastures at mating group. Similarly, fetal blood thyroid hormone levels were not affected by treatment. In terms of fetal thyroid morphology, changing either from or to sewage sludge-treated pasture at mating was associated with a reduction in the number of thyroid follicles. Variable effects were observed in terms of blood vessel number, together with areas of immature thyroid follicle

development, reduced NIS (sodium-iodide-symporter) expression and focal lack of mature angio-follicular differentiation in continuously exposed group fetal females and sewage sludge moved to control group fetal males being most affected. In contrast, in the Windows study (Lea et al., 2016), fetal thyroid weight was reduced in the late exposure window, together with reduced free T3 and increased free T4 in the same group.

4.4. Adverse consequences of sewage sludge exposure in adulthood (18 months)

Fetal and post-natal sewage sludge exposure affected male lamb bones more than the females (12/23 bone parameters significantly different in males vs 2/23 parameters affected in females). In general, the bones of sewage sludge-exposed animals were more fragile compared to the controls (Lind et al., 2009b). In contrast, fetal bones were markedly less affected by the EDs present in sewage sludge pastures, unlike their mothers who showed reduced mineralisation and cross-sectional areas of some bones (Lind et al., 2010).

Testes from *in utero* and post-natally sewage sludge-exposed rams were analysed at age 18 months old (Bellingham et al., 2012) and although there was no effect on mean testis weights, a subset of exposed rams had markedly lighter testes. While there were no differences in total mean number of germ cells, 42% of the exposed rams had total number of germ cells were lower than the minimum numbers in the control group. When this subgroup was analysed separately the number of germ cells per testes, germ cells per Sertoli cell, germ cell absolute volume, number of round spermatids, number of spermatocytes were all reduced by sewage sludge exposure. They also exhibited an increase in tubule number, number of tubules with dilated lumens and an increase in Sertoli cell only tubules which lacked germ cells in the testes collected from biosolids exposed animals. The sewage sludge-exposed rams with small testes also had lowered FSH. While the *in utero* + post-natally exposed adult animals (Bellingham et al., 2012) showed different consequences of sewage sludge-exposure than the *in utero* exposed fetuses (Paul et al., 2005), changes observed in both studies, especially the reduction in the number of fetal Sertoli cells and adult Sertoli cell-only tubules and the reduction in germ cells in a subset of the adults, would disrupt sperm producing capacity. Analysis of ovaries from our study of *in utero* + post-natally sewage exposed 18 month old ewes has not currently been published. However,

(Fowler, personal communication), the consequences of the sewage sludge exposure include adverse morphological and molecular markers of ovarian function and health. One striking aspect is the retention of some fetal ovarian characteristics in the sewage sludge-exposed group.

In Filis et al. (Filis et a., 2019), we investigated the livers from the adult animals discussed in the preceding paragraph. These animals exhibited sexually dimorphic patterns of ED burdens as discussed at the start of this section. The life-long sewage sludge-exposure of these animals manifested as altered xenobiotic and detoxification responses. The liver proteomes of both sexes were disrupted by sewage sludge exposure and proteins with altered expression profiles included major plasma-secreted and blood proteins, and metabolic enzymes. Pathway analysis predicted dysregulation of cancer-related pathways and altered lipid dynamics. Follow-up confirmed these predictions, with a reduction in total lipids in female livers and up-regulation of cancer related transcript markers in male livers respectively by sewage sludge exposure.

To draw together the sheep sewage sludge model section, this complex picture of highly variable and sometimes inconsistent changes in ED levels in the animals are reminiscent of the data held in human exposure cohorts, such as NHANES (National Health & Nutrition Survey: https://www.cdc.gov/nchs/nhanes/index.htm). In addition, this variable ED exposure profile is highly likely to contribute to the variable tissue- and sex- specific morphological, endocrine and molecular marker alterations observed in the sewage sludge exposed fetuses and adult offspring. In many ways this variability is very similar to data seen in human studies.

5. CONCLUSION

The complexity of the regulatory pathways triggered by EDs underlines the need for the identification of systemic biomarkers that can be robustly linked to ED exposures with minimal possible uncertainty. Choosing the right model is one of the best ways to reduce uncertainties. Rodents have proven to be very useful in evidencing the potential of chemicals to induce endocrine disruption and to provide data on their potential mechanisms of action and associated adverse effects. However, because the endocrine system is very plastic, it is clear that it is necessary to integrate the notion of interspecies differences into our way of thinking with respect to EDs. Sheep studies have clearly established that non-rodent

mammalian models contribute strongly to a better understanding of human health risk assessment than necessarily achieved using classical rodent studies. It should be acknowledged however, that like every other model, the large animal models, including sheep, also exhibit some limitations. Many of these limitations are practical, reflecting the need for large scale animal facilities. Sheep have long gestation lengths, making them more similar to human from the standpoint of duration of exposure and usually the timelines of ontogenesis. The price to pay, however, is that experiments can take years to complete. Sheep are, therefore, not suitable for rapid screening studies. Sheep placentation has some differences from the human and may not accurately predict human fetal exposure. However, the rodent placenta is not any better from this point of view and rodent models also do not allow kinetic investigations required for modelling predictive approaches. Furthermore, investigators do not have access to as many tools for sheep as for rodents, in particular molecular ones for mechanistic investigations. Finally, for some endpoints of adverse effects, such as cognitive or behavioural alterations, there are almost no well standardised tools or validated sheep models for human neurobehavioural abnormalities.

Because of its relevance to human physiology, we conclude that the sheep model and more generally large animal models can take us one step further for a better understanding of EDs. But because of their limitations in particular technical ones, those models have to be seen as complementary to rodent models. Anyway, the best possible models should be used to address scientific questions and it is our responsibility to question which model/models is/are the most precise to give the right answers to each specific question for a better understanding and prediction of ED adverse effects.

704 **FIG. LEGENDS**

705 **Fig. 1**: Transplacental exchange rates of BPA, BPS and their main glucuronidated metabolites. The %
706 represent the fraction of a given dose that crosses the placental barrier or the rate at which the parent
707 molecules are metabolised into glucuronidated conjugates, or, alternatively, the reactivation rate of the
708 conjugate into native molecules. These data were generated in an ovine model of catheterised fetus at a
709 late gestational stage.

710

711 **Fig. 2:** Possible interactions of endocrine disruptors with extra-thyroidal metabolism of thyroid
712 hormones. The thyroid gland synthetises and secretes both T4 and the more biologically active T3.
713 Thyroid secretion accounts for only 20% of circulating T3. Once in the blood more than 99% of the
714 TH are bound to proteins. Two of these proteins, thyroid binding globulin (TBG) and transthyretin
715 (TTR) are of high affinity and specificity. The TH-bound fraction is in equilibrium with the free
716 hormone fraction, the only accessible one for the liver and the target tissues. Once in the liver, TH can
717 be deiodinated by specific deiodinases (DOIs) or metabolised mainly through conjugation reactions by
718 UGTs or sulphotranferase enzymes. The expression of these enzymes can be induced following the
719 activation of xenosensor nuclear receptors (XNRs) thus leading to increased clearance of TH.

720

721 **Fig. 3**: Pharmacological properties of the two specific binding proteins transthyretin (TTR) and thyroxin
722 binding globulin (TBG) of thyroid hormones (TH) in both humans and sheep. Kd is the dissociation
723 constant that characterises the affinity of the protein for T4, the lower Kd, the higher affinity of the
724 binding proteins for TH.

725

726 **Fig. 4:** Timelines of fetal thyroid axis maturation and neural development in sheep (Barlow, 1969;
727 Ferreiro et al., 1987; Fisher, 1991; Fisher et al., 1976; McIntosh et al., 1979; Polk et al.,
728 1991; Thomas et al., 1993), humans (Howdeshell, 2002) and rats (Howdeshell, 2002) from
729 conception to birth or to PND22 for the rat. Only global starting points of the different processes are
730 indicated, there is no indication about the quantitative changes of the different parameters throughout
731 fetal development (except for the fetal thyroid function). All these processes keep evolving during

subsequent postnatal development. The human and rat elements are adapted from Howdeshell; 2002.

Fig. 5: Schematic summary of the experimental scheduling used in the Aberdeen sewage sludge studies. Days of gestation along the top between "sex determination" and "birth". Animals were harvested at 80, 110, 140 days of gestation and at 18 months. While the *Windows* and *Cross-over* studies were single large studies, the *Always* study where animals were not changed from pre-gestation exposure conditions (sewage sludge vs control [inorganic] fertiliser) was run several times.

Fig. 6: Global summary of the findings of the Aberdeen sewage sludge studies. Processed human sewage sludge pellets were used according to agricultural guidelines. There was accumulation of EDs in the soil but only modest changes in maternal and fetal tissue levels of EDs. Nevertheless, extensive morphological, endocrine, molecular and behavioural consequences for the fetus, some of which have been shown to persist into adulthood. *personal communication P.A. Fowler, University of Aberdeen, manuscript in preparation. For references see relevant text.

748 **Competing interest statement**

749 **None of the authors has competing interests.**

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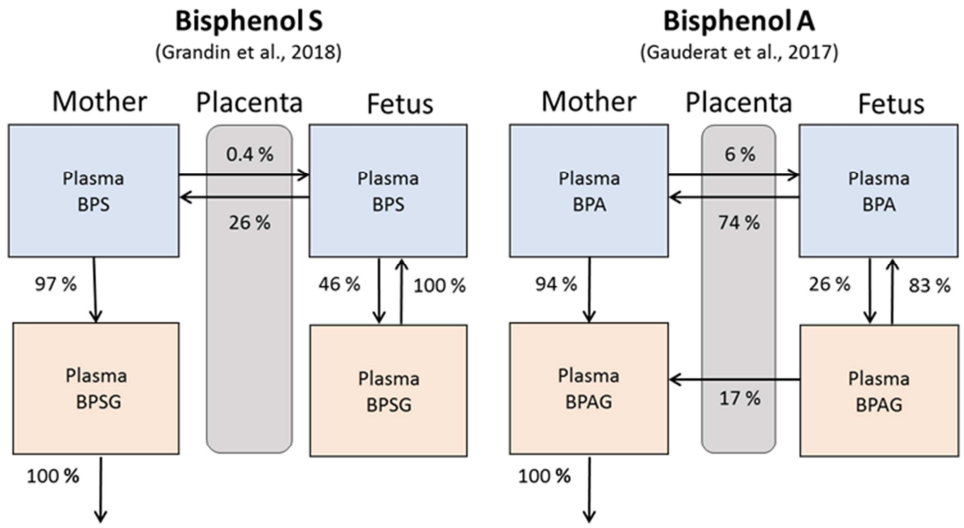

Figure 1

Comment citer ce document :
Viguié, C. (Auteur de correspondance), Chaillou, E., Gayrard, V., Picard-Hagen, N., Fowler, P. A. (2020). Toward a better understanding of the effects of endocrine disrupting compounds on health: Human-relevant case studies from sheep models. Molecular and Cellular Endocrinology, 505. . DOI : 10.1016/j.mce.2020.110711

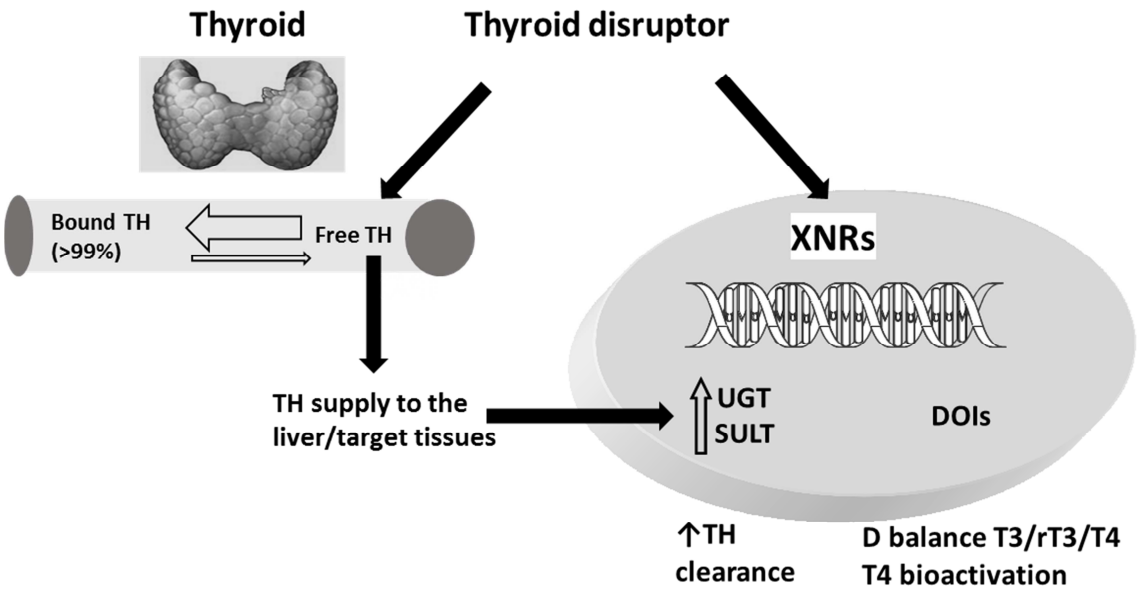

Figure 2

TBG	Concentration	270 nM	160 nM
	Kd	0.11 nM	0.11 nM
TTR	Concentration	2952 nM	4621 nM
	Kd	7 nM	6 nM

Figure 3

Comment citer ce document :
Viguié, C. (Auteur de correspondance), Chaillou, E., Gayrard, V., Picard-Hagen, N., Fowler, P. A. (2020). Toward a better understanding of the effects of endocrine disrupting compounds on health: Human-relevant case studies from sheep models. Molecular and Cellular Endocrinology, 505. . DOI : 10.1016/j.mce.2020.110711

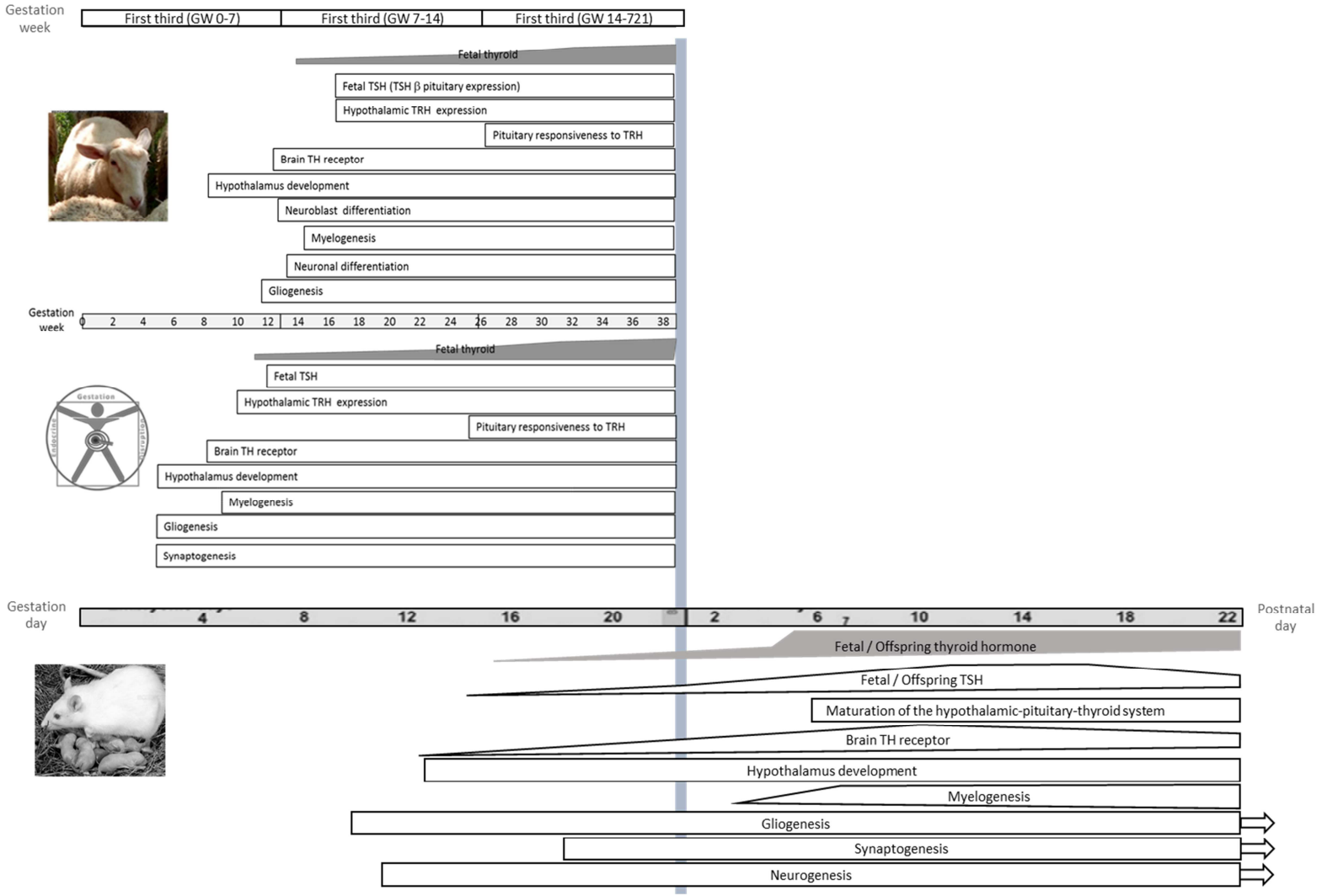

Figure 4

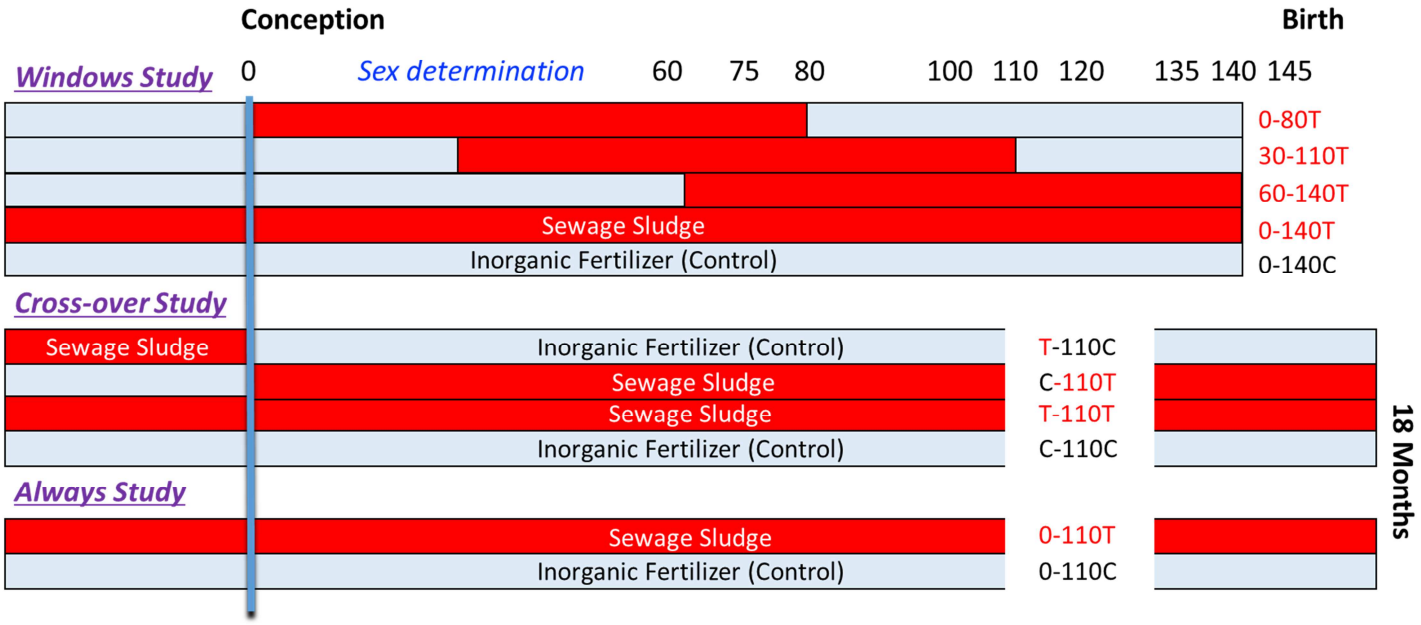

Figure 5

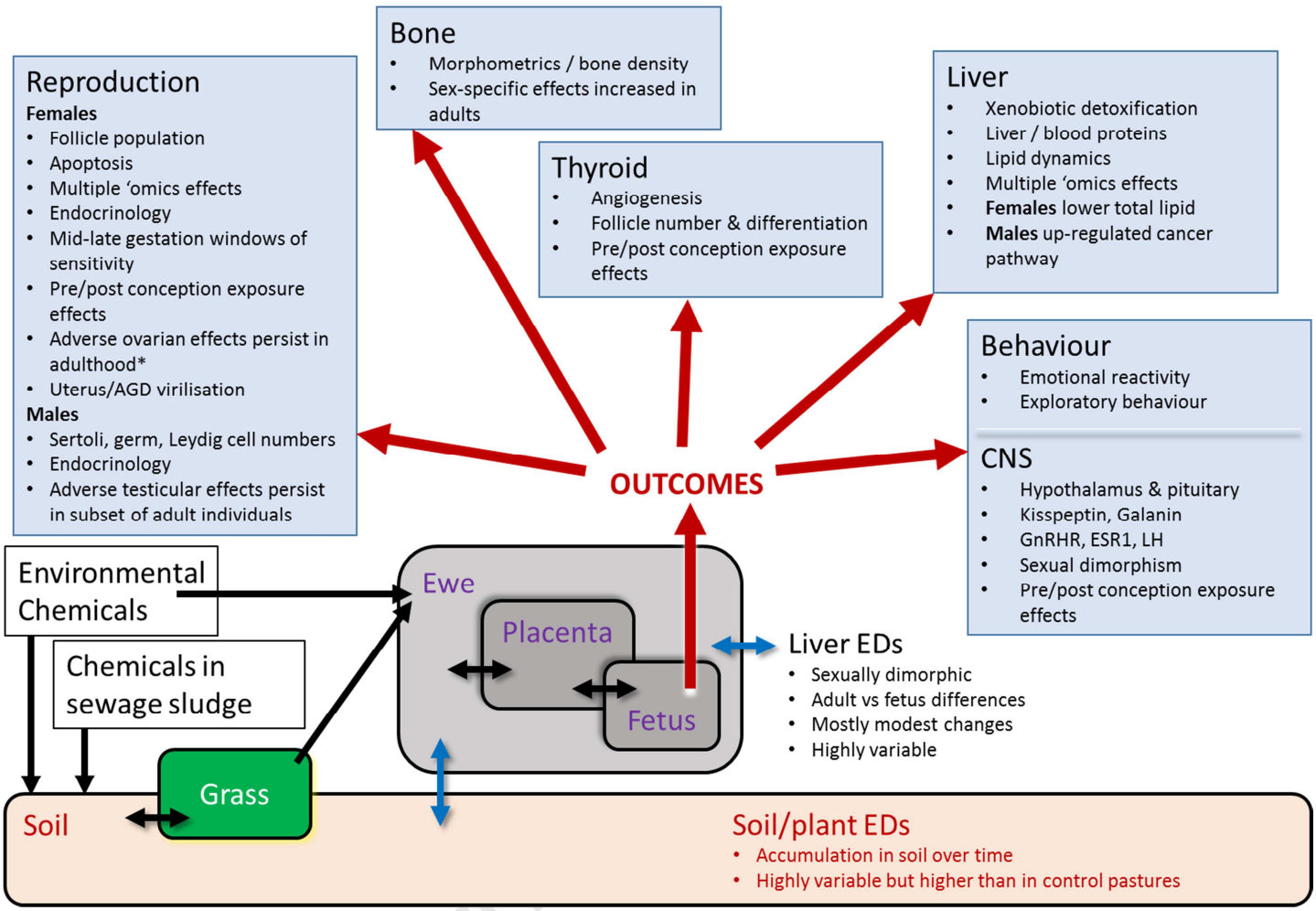

Figure 6

Viguié et al., Highlights

- Endocrine disruption is a very complex and critical public health issue
- There is a need for human-relevant models to assess endocrine physiology and the physiology of period at risks
- The sheep is a good alternative to classical rodent models to understand fetal exposure, thyroid disruption and its consequences on brain development
- The sheep allows real-life exposure scenario to complex mixtures.

Author contributions

Catherine Viguié coordinated the manuscript preparation.

All author equally contributed to the redaction according to their own field of competences :

- C Viguié: introduction – thyroid and brain -conclusion
- Elodie Chaillou: relevance of the sheep for brain development
- Véronique Gayrard and Nicole Picard-Hagen: fetal exposure
- Paul Fowler: the sheep a model fitted for real life scenario exposure.

All authors have contributed to raise different fundings covering their investigations the results of which have been used for this review.