Author/s:
Li, D;Donnelley, M;Parsons, D;Habgood, MD;Schneider-Futschik, EK

Title:
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Date:
2024-08-01

Citation:
Li, D., Donnelley, M., Parsons, D., Habgood, M. D. & Schneider-Futschik, E. K. (2024). Extent of foetal exposure to maternal elexacaftor/tezacaftor/ivacaftor during pregnancy. British Journal of Pharmacology, 181 (15), pp.2413-2428. https://doi.org/10.1111/bph.16417.

Received: 24 October 2023 | Revised: 28 March 2024 | Accepted: 28 March 2024

DOI: 10.1111/bph.16417

RAPID COMMUNICATION

Extent of foetal exposure to maternal elexacaftor/tezacaftor/ivacaftor during pregnancy

Danni Li[1] | **Martin Donnelley**[2,3,4] | **David Parsons**[2,3,4] | **Mark D. Habgood**[1] | **Elena K. Schneider-Futschik**[1]

[1]Department of Biochemistry & Pharmacology, School of Biomedical Sciences, Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne, Parkville, VIC, Australia

[2]Robinson Research Institute, University of Adelaide, Adelaide, South Australia, Australia

[3]Adelaide Medical School, University of Adelaide, Adelaide, South Australia, Australia

[4]Department of Respiratory and Sleep Medicine, Women's and Children's Hospital, North Adelaide, South Australia, Australia

Correspondence
Elena K. Schneider-Futschik, Department of Biochemistry & Pharmacology, School of Biomedical Sciences, Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne, Parkville, VIC 3010, Australia.
Email: elena.schneider@unimelb.edu.au

Funding information
National Health and Medical Research Council, Grant/Award Number: APP1157287; Cystic Fibrosis Foundation, Grant/Award Number: DONNEL21GO; Australian Cystic Fibrosis Research Trust

Abstract

Background and Purpose: Cystic fibrosis (CF) patients are living longer and healthier due to improved treatments, e.g. cystic fibrosis transmembrane conductance regulator (CFTR) modulator therapy elexacaftor/tezacaftor/ivacaftor (ETI), with treatment possibly occurring in pregnancy. The risk of ETI to foetuses remain unknown. Thus the effect of maternally administered ETI on foetal genetic and structural development was investigated.

Experimental Approach: Pregnant Sprague Dawley rats were orally treated with ETI (6.7 mg·kg^{-1}·day^{-1} elexacaftor + 3.5 mg·kg^{-1}·day^{-1} tezacaftor + 25 mg·kg^{-1}·day^{-1} ivacaftor) for 7 days from E12 to E19. Tissue samples collected at E19 were analysed using histology and RNA sequencing. Histological changes and differentially expressed genes (DEG) were assessed.

Key Results: No overt structural abnormalities were found in foetal pancreas, liver, lung and small intestine after 7-day ETI exposure. Very few non-functionally associated DEG in foetal liver, lung and small intestine were identified using RNA-seq. 29 DEG were identified in thymus (27 up-regulated and two down-regulated) and most were functionally linked to each other. Gene ontology enrichment analysis revealed that multiple muscle-related terms were significantly enriched. Many more DEG were identified in cortex (44 up-regulated and four down-regulated) and a group of these were involved in central nervous system and brain development.

Conclusion and Implication: Sub-chronic ETI treatment in late pregnancy does not appear to pose a significant risk to the genetic and structural development of many foetal tissues. However, significant gene changes in foetal thymic myoid cells and cortical neuronal development requires future follow-up studies to assess the risk to these organs.

KEYWORDS
cystic fibrosis, elexacaftor/tezacaftor/ivacaftor, foetal, pregnancy

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Abbreviations: AB/PAS, Alcian blue/periodic acid-schiff; CF, cystic fibrosis; DEG, differentially expressed gene; ETI, elexacaftor/tezacaftor/ivacaftor; F508del, Phe508del variant; GO, gene ontology; H&E, haematoxylin and eosin; Log2FC, Log2 fold change; RNA-seq, RNA sequencing.

1 | INTRODUCTION

Breakthroughs in the development of therapies that target the underlying cause of cystic fibrosis (CF) were made possible by the discovery that CF is caused by pathogenic variants of **the cystic fibrosis transmembrane conductance regulator (CFTR) gene** (Bell et al., 2020; Parkins et al., 2018; Turcios, 2020). Highly effective CFTR modulator therapies (HEMT) such as **elexacaftor**/**tezacaftor**/**ivacaftor** (ETI) have led to unprecedented improvements in lung function and other clinical outcomes including fertility in eligible patients, transforming the landscape of clinical care for people with CF (Heltshe et al., 2017; Kazmerski et al., 2017; Taylor-Cousar & Jain, 2021). This is reflected in the dramatic increase in the number of pregnancies to people with CF in the United States and worldwide (Cystic Fibrosis Foundation, 2023). However, for pregnant women and breastfeeding mothers, ceasing ETI therapy may cause sudden and serious decline in lung conditions and risk their own health as well as the health of their babies (Trimble & Donaldson, 2018). Hence, it is often in the best interest of mothers to continue ETI therapy during pregnancy and while breastfeeding.

CFTR modulators can directly interact with dysfunctional CFTR proteins to either (i), potentiate CFTR channel opening probability (potentiator) or (ii) enhance CFTR trafficking to the cell surface (corrector) (Rowe & Verkman, 2013). Just over 10 years ago, the potentiator ivacaftor became the first CFTR modulator to be approved for people with CF. More recently, combining the correctors elexacaftor and tezacaftor with the potentiator ivacaftor resulted in a major treatment breakthrough by producing robust improvements in lung function and sweat chloride in people with CF that have at least one copy of F508del (US Food and Drug Administration, 2019). ETI is approved for people with CF over 2 years and over 6 years in the United States and Australia, respectively (European Medicines Agency, 2020; Gabillard-Lefort et al., 2022; Heijerman et al., 2019; Ramsey & Bell, 2022; Wainwright et al., 2023; Zemanick et al., 2021). Although clinical trials of CFTR modulators did not include pregnant women, it is anticipated that approximately 90% of pregnant women with CF will be eligible for modulator therapies in the next decades (Heijerman et al., 2019; Middleton et al., 2019).

Exposure to CFTR modulators has unknown impact on foetal growth. Hence understanding the safety toxicology data of ETI in the context of pregnancy is critical (Ramsey & Bell, 2022). Animal reproduction studies have shown that each component of ETI is transferred across the placenta, albeit none were associated with obvious teratogenicity or adverse developmental effects in foetal animals (US Food and Drug Administration, 2019). Noteworthy, these studies were conducted with single modulators only and not as the combination therapy used clinically. However, previous work has shown that CFTR modulators can have drug–drug interactions that affect pharmacokinetic properties and hence therapeutic outcomes (Hanafin et al., 2021). Consequently, preclinical prenatal safety studies of concomitant use of combination therapies are urgently needed.

Clinically, more than 50 cases of unplanned pregnancies have been reported where ETI treatment was continued during pregnancy (Collins et al., 2022; Fortner et al., 2021; Gómez-Montes et al., 2023; Kendle et al., 2021; Szentpetery et al., 2022; Taylor-Cousar & Jain, 2021). In a study following five pregnancies under ETI, no neonatal complications were observed (Table 1) (Kendle et al., 2021). In a case series covering 45 pregnancies under ETI, five minor foetal complications from three infants were reported, but their relatedness to maternally administered ETI could not be determined (Table 1) (Taylor-Cousar & Jain, 2021). First trimester miscarriage rate within this series did not increase following ETI exposure, compared with that in general population. Mild elevations of alanine transaminase (ALT) were observed in one infant after ETI exposure, which was ultimately resolved (Collins et al., 2022). However, recent case reports have also revealed that in utero ETI treatment successfully reduced clinical presentations in babies with CF (Collins et al., 2022; Fortner et al., 2021; Gómez-Montes et al., 2023; Szentpetery et al., 2022**)**. Overall, the small number of foetal complications may suggest that ETI is safe during pregnancy, although the reliability of this conclusion is undermined by incomplete animal reproductive studies and limited individual case reports. There is currently an ongoing multicentre study investigating the impacts of ETI on infants after exposure in pregnancy and lactation, and results should be reported within following years (Jain et al., 2022).

With the rapid increase in the number of pregnancies and our previous work highlighting widespread ETI accumulation in different foetal tissues, understanding the potential impacts on prenatal development following maternally administered ETI is urgently needed (Li et al., 2024). Herein, we investigated impacts of exposure to ETI on foetal tissue structural development and gene expression changes using a pregnant rat model, and demonstrated that ETI was potentially safe to many foetal tissues but may adversely affect foetal thymic and brain development. Rats were chosen as experimental subjects due to their size, technical manipulation, airway characteristics resembling those of human and the availability of more ETI pharmacokinetic data in pregnant rats (US Food and Drug Administration, 2019; Widdicombe et al., 2001).

2 | METHODS

2.1 | Animals

Time-mated pregnant Sprague–Dawley rats were supplied by The University of Melbourne. Rats were housed in single cages from embryonic day (E) 12 with a 12-h light/dark cycle and supplied with *ad libitum* access to water and standard food (a fixed dry pallets formulation designed for rats from Specialty Feeds, Western Australia). All animal experiments were approved by The University of Melbourne Animal Ethics Committee (Ethics ID: 10393), were conducted in accordance with National Health and Medical Research Committee guidelines and are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

TABLE 1 Case reports of elexacaftor/tezacaftor/ivacaftor in pregnancy.

Case report	Kendle et al., 2021					Taylor-Cousar & Jain, 2021
Genotype	**F508del/R1066C**	**F508del/P205S**	**F508del/F508del**	**F508del/G551D**	**F508del/C3791del**	**NA**
Time of starting ETI	4 months prior	2 months prior	1 month prior	Same month of delivery	1 month prior	41 prior to pregnancy, 4 from 2nd 3rd trimesters
Trimester exposed	1–3 (stopped at 6 weeks and restarted in 31 weeks)	1–3	1–3	1–3	1–3	1–3 ($n = 23$)[a]
Infant phenotype	Non-CF	Non-CF	Non-CF	Non-CF	Non-CF	All non-CF
Infant complications	No	No	No	No	No	Unknown relationship to ETI: 1 mild congenital malformation, 1 choroid plexus cyst, uretocele and transient transaminitis 1 low set ears
Maternal complications	Elevation of serum transaminases	Intrahepatic cholestasis	No	No	2 antenatal exacerbations 1 Transaminitis (postpartum day 1)	Related to ETI: 1 cholecystits Unknown relationshi.p to ETI: 1 obstetric cholestasis, 1 pulmonary exacerbation

[a] Six discontinued at the time of diagnosis, four first trimester miscarriage during ETI treatment, two first trimester miscarriage after discontinuation of ETI, three electively aborted, seven ongoing pregnancies at the time of repo.

TABLE 1 (Continued)

Case report	Collins et al., 2022 (subject 3 was firstly reported in Fortner et al., 2021)			Szentpetery et al., 2022	Gómez-Montes et al., 2023
Genotype	**F508del/F508del**	**F508del/F508del**	**F508del/F508del**	**Healthy (F508del carrier)**	**Healthy (F508del carrier)**
Time of starting ETI	2nd trimester	Prior	Prior	32 weeks	31 + 1 weeks
Trimester exposed	2–3	1–3	1-3	3	3
Infant phenotype	Non-CF	Non-CF	CF (F508del/F508del)	CF (F508del/F508del)	CF (F508del/F508del)
Infant complications	Temporary cardiac murmur (self-resolved)	Temporary alanine transaminase elevation	CF but normal newborn screening, normal pancreatic function and lower-than-expected sweat chloride levels	CF, but normal pancreatic elastase and transaminase level	CF, abnormal but higher-than-expected pancreatic elastase, no bowel obstruction
Maternal complications	No	Maternal cholecystitis	No	Occasional headaches	No (a skin rash not likely related to ETI)

[a] Six discontinued at the time of diagnosis, four first trimester miscarriage during ETI treatment, two first trimester miscarriage after discontinuation of ETI, three electively aborted, seven ongoing pregnancies at the time of repo.

2.2 | Long-term treatment with elexacaftor/tezacaftor/ivacaftor (ETI)

Pregnant rats were randomly assigned into two groups:- an ETI-treated group and a control group. Rats in the ETI-treated group were orally administered a dose of ETI equivalent to a human clinical dose (6.7 $\mathrm{mg \cdot kg^{-1} \cdot day^{-1}}$ elexacaftor + 3.5 $\mathrm{mg \cdot kg^{-1} \cdot day^{-1}}$ tezacaftor + 25 $\mathrm{mg \cdot kg^{-1} \cdot day^{-1}}$ ivacaftor) for 7 days from embryonic (E) day 12 to E19. To match the human dose, ivacaftor was delivered orally in food treats every 12 h (twice daily), and tezacaftor and elexacaftor were delivered once every day (only in the morning dose). Feeding was visually observed to confirm ingestion. All pregnant rats were monitored during the treatment period to identify any abnormalities in weight, appearance, condition or behaviour. E12 was selected as the earliest day when pregnancy can be confirmed. E19 was selected as the foetal rats at this stage are of sufficient size to obtain samples from individual animals for analysis without pooling. According to previous research, CFTR modulator treatment over 4 days allows the components of drug to reach steady state in plasma, so our 7-day treatment is long enough to provide stabilized ETI concentrations in foetuses (Choong et al., 2022).

2.3 | Foetal tissue collection

All pregnant rats were deeply anaesthetized with intraperitoneal (i.p) injection of 25% urethane (0.7–1 ml/100 g body weight) at E19. An endotracheal cannula was inserted prior to foetal collections to maintain the airway. Pups with foetal movements and a clear colour distinction between blood in the umbilical veins and arteries were selected for tissue collection. Foetuses were sequentially dissected and samples of foetal lung, small intestine (duodenum, jejunum and ileum), liver, thymus and cortex were flash frozen and stored at −80°C. Tissue samples from six dams (three drug-treated + three controls) were collected for RNA analysis to make three biological replicates in each treatment group. For each foetal tissue sample, tissue from one male pup and one female pup (in the same litter) were pooled together to average out the gender-related gene expression differences. Only one male and one female pup were randomly selected from each litter to minimize interlitter effect. Dissected small intestine (jejunum), pancreas, liver, lungs, thymus and cortex were also immersion fixed in Bouin's fixative immediately at room temperature for histology. The same part of each tissue was captured from each pup for consistency.

2.4 | RNA extraction, sequencing and analysis

Total RNA was extracted from weighed aliquots of macerated tissues using the RNeasy Plus Mini Kits (Qiagen) according to the manufacturer's protocol. Extracted RNA samples were dissolved in RNase-free water and the RNA content measured using a nanodrop. Only samples with A260/A280 ratios greater than 2.0 and A260/A230 ratios between 2.0 and 2.2 were selected for RNA sequencing. An Illumina double stranded mRNA library was prepared by Australian Genome Research Facility (AGRF) and only samples that passed their quality control checks were sequenced.

The Galaxy Australia platform and online software packages were used to identify differentially expressed genes (DEG) from sequenced RNA. RNA data were processed through Trimmomatic (Galaxy Version 0.36.6) to cut bases off the start and end of a read if below a minimum quality of 20. All sequence reads were then mapped to the reference genome, Rat Jul. 2014 (RGSC 6.0/rn6) using HISAT2 with the paired, reverse stranded setting (Galaxy Version 2.2.1 + galaxy1). Mapped reads were assigned to genes based on the rn6 genome annotation file extracted from the Ensembl genome database using featureCounts (Galaxy Version 2.0.3 + galaxy1). Assigned genes were then processed by DEseq2 (Galaxy Version 2.11.40.7 + galaxy2) to identify genes differentially expressed between the treated and control groups. DEseq2 is an overly conservative differential expression tool with high sensitivity designed for small studies with few replicates, allowing a more general, data-driven parameter estimation, hence more suitable for our datasets (Love et al., 2014; McDermaid et al., 2019; Seyednasrollah et al., 2015). Differentially expressed genes (DEG) were defined as those with an adjusted P value <0.05 (the Benjamini and Hochberg's approach) and false discovery rate (FDR) > 2. Corresponding gene symbols and names were generated through annoateMyID (Galaxy Version 3.16.0 + galaxy1). Volcano plots were generated,and DEG were highlighted in red (up-regulated) or blue (down-regulated). Gene ontology (GO) enrichment analysis was performed via goseq (Galaxy Version 1.50.0 + galaxy0) to unravel significantly enriched terms with *a*djusted $P < 0.05$. STRING database (Version 12.0) was then used to identify any protein–protein interactions (PPI) between DEG.

2.5 | Histology

Tissues were immersion fixed in Bouin's fixative for 24 h, then dehydrated in increasing concentrations of ethanol over 3–5 days, cleared overnight in chloroform and embedded in paraffin. Ribbons of 5-μm-thick sections were cut from each paraffin block using a Leica RM2125 RTS microtome, stained with haematoxylin–eosin (H&E) (pancreas, liver, lungs, thymus and cortex) or Alcian blue periodic acid Schiff (AB/PAS) (small intestine) (AB/PAS staining was processed by Melbourne University Histology Platform). Whole brains were paraffin-fixed and prepared for sectioning in the olfactory lobe to cerebellum direction. This ensured that we could capture the intact coronal sections of cortex with clear morphological features of different layers. Similar cortical planes were selected for all pups for consistency. Stained sections were examined and digital images were captured with an Olympus BX50 light microscope fitted with an Olympus DP 71 digital camera.

2.6 | Histological analysis of lung tissue

Lung section images were analysed using Image J 1.53k software. The lung open airspace areas (mm^2) excluding large bronchi were quantified and the total open-air space/total lung area ratios were calculated. For each lung tissue, eight sections were randomly selected for measurement.

2.7 | Histological analysis of intestinal tissue

Longitudinal intestinal villi sections were imaged and processed through Image J 1.53k. AB/PAS-positive goblet cells along the villi were mainly located near the base of the villi with very few near the tips (Figure S1). This coupled with frequent bending of the tips out of the plane of section and damage to some tips made it difficult to quantify all positive cells on each villus. To minimize those effects, 30 intestinal villi not exhibiting significant mechanical damage were randomly selected in each jejunum section and the average villi width (AW) were calculated. For each villus, a length standardized at equivalent to three times the AW measured up from the base of the villus, was used to define the region in which positive stained goblet cells were counted.

2.8 | Histologic analysis of cortex

Cortical section images were analysed using Image J 1.53k software. Four cortical layers were identified:- marginal zone (MZ), cortical plate (CP), intermediate zone (IZ) and ventricular zone (VZ) (Figure S2). The layers in the middle cortical area were not very apparent as E19 age, hence in this study we analysed the section between cortical plate and ventricular zone as one IZ to minimize any bias. Mean grey values were measured to assess the cell density in each cortical layer. To minimize the effect of staining variability, we calculated the relative mean grey value (%) (intermediate zone mean grey value/ cortical plate mean grey value and intermediate zone mean grey value/ ventricular zone mean grey value). For the measurement of thickness, we measured cortical thickness at two different locations for every pup (Figure S3). For each location, we compare the relative thickness between pups in both groups. We also normalized each layer to the total thickness (%) to minimize the effect of foetal brain size variability.

2.9 | Statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2022). Histological and RNA-seq analysis were conducted blind to the treatment group. All graphs were generated using GraphPad Prism version 9 and data were presented as mean ± standard deviation (SD). Unpaired Student's *t* test (between two groups) or one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test (between multiple groups) were performed as the post hoc test. A *P* value <0.05 was considered statistically significant.

2.10 | Materials

Elexacaftor, tezacaftor and ivacaftor (ETI) were purchased from ???? (address), while urethane was obtained from Sigma (address). Details of other materials and suppliers are provided in the specific sections.

2.11 | Nomenclature of Targets and Ligand

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/24 (Alexander et al., 2023).

3 | RESULTS

3.1 | Subchronic ETI (elexacaftor/tezacaftor/ivacaftor) exposure in late pregnancy does not adversely affect the structural development of multiple foetal tissues

Examination of H&E-stained sections of pancreas and liver did not reveal any apparent structural abnormalities at this late E19 gestational age between the two groups (Figure 1a–d). Similarly, the lung tissues of ETI-treated E19 pups did not reveal any overt histopathology, compared with the control ones (Figure 1e,f). The average air space opening showed no significant difference between the ETI-treated (32.66 ± 5.25%) and control (30.99 ± 3.07%) groups (Figure 1k), with similar pulmonary branching and interstitial wall thickness. No signs of pulmonary inflammation were apparent in either group. The histology of thymus of ETI-treated pups also displayed no histopathological abnormalities when compared with control ones (Figure 1g,h). Jejunum were stained with AB/PAS and mucus-containing cells displayed as dark pink and purple dots were visualized (Figure 1i,j). Compared with control groups, the average number of AB/PAS-positive goblet cells per villus in jejunum was not significant affected by 7 days of ETI treatment (Treated, 2.88 ± 0.56 cells vs. Control, 2.61 ± 0.36) (Figure 1l). No irregular goblet cell shapes were observed in the ETI-treated group. Together, these results suggested that 7 days exposure to maternally administered ETI did not adversely impact foetal structural development of the pancreas, liver, lung, thymus and intestinal goblet cells.

Histologically, foetal cortex seemed to develop normally in both groups (Figure 2a,b). The relative mean grey value of IZ normalized to the mean of CP (Treated, 124.95 ± 3.75% vs. Control, 122.66 ± 2.73%) and VZ (Treated, 140.92 ± 8/99 vs. Control, 137.20 ± 5.27%) appeared no significant difference in both groups (Figure 2c,d). For all four layers, the relative thickness measured at both locations displayed no significant difference between two groups (Figure 2e,f). Those results suggested that there were no alterations in cell density nor size of cortical layers after the drug exposure.

3.2 | RNA-seq analysis of multiple foetal tissues at E19 age

RNA sequencing was performed in triplicate using tissue samples from separate foetuses in both the ETI-treated and control groups. Differentially expressed genes (DEG) in ETI-treated pups compared with control pups were identified and presented in the following

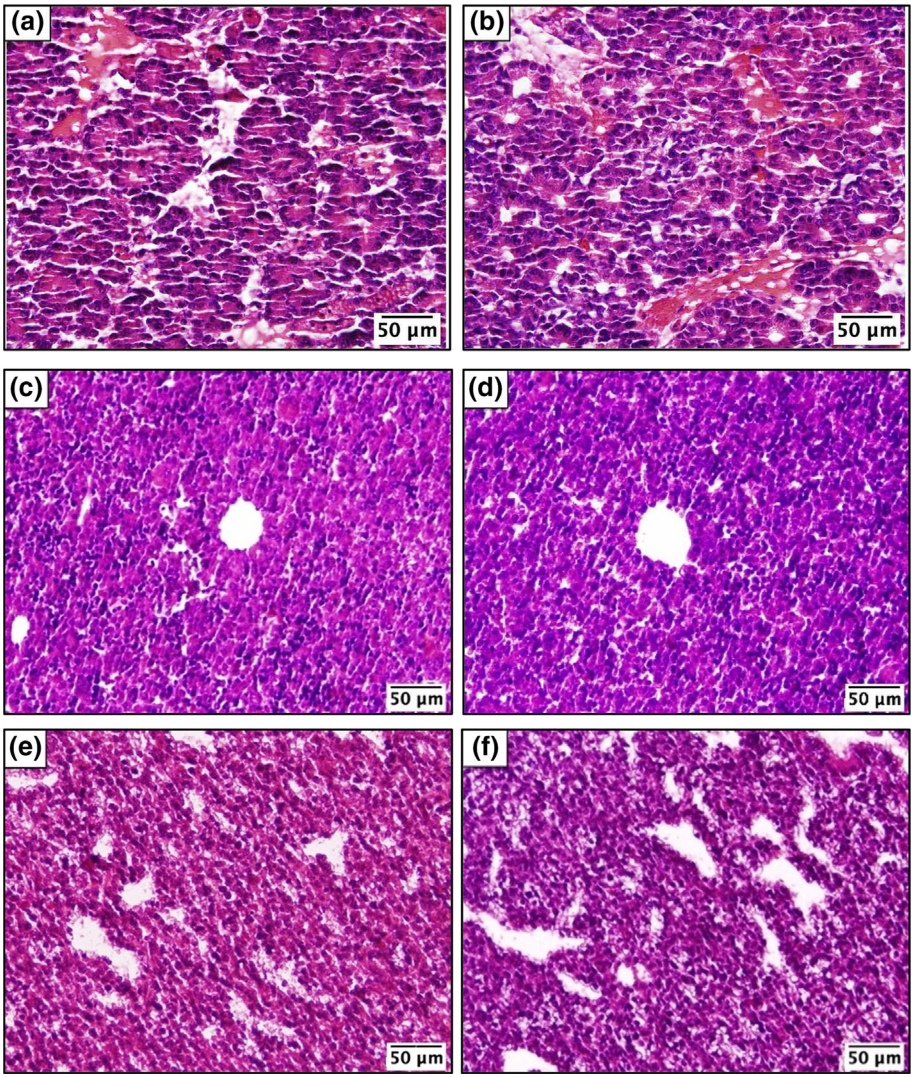

FIGURE 1 Histological analysis of different tissues in E19 pups after subchronic elexacaftor/tezacaftor/ivacaftor exposure. (a–h) Representative H&E-stained histological sections of pancreas (a,b), liver (c,d), lung (e,f), thymus (g,h) and small intestine (i,j) from the ETI-treated (a,c,e,g,i) and control (b,d,f,h,j) E19 pups. Scar bar = 50 or 100 μm as indicated. (k) Comparison of the percentage of lung air space. Lung air space area % = total open air space area/total lung area (excluding large bronchi). Each point represents a single randomly chosen section within one lung sample. Each scattered column represents lung sections from the same pup. Eight sections were analysed for each lung sample. In each group, points with the same shape represent pups from the same litter. (l) The average number of AB/PAS-positive goblet cells per villus in jejunum of E19 rat pups. Each data point represents the AB/PAS-positive cell count of one pup averaged from 25 randomly selected villi. Data were analysed by unpaired t test, presented as mean ± SD (k,l). n = 5 (i,j,l), 6 (g,h) or 8 (a–f,k) pups per group (pups in each group were from one to three different litters). AB/PAS, Alcian blue/periodic acid-Schiff; ETI, elexacaftor/tezacaftor/ivacaftor; E, embryonic day; H&E, haematoxylin & eosin.

volcano plots and tables. We further performed protein–protein interactions and gene ontology (GO) enrichment analysis to elucidate the functional relevance of the DEG and associations between their encoded proteins.

3.2.1 | Liver

In the liver, we characterized two significantly up-regulated genes with moderate fold change (approximately threefold to eightfold) in the ETI-treated group: *Asb15* and *Gdf15* (Table 2) (Figure 3a). However, no enriched gene ontology (GO) terms nor functional associations between DEG-encoded proteins were identified.

3.2.2 | Lung

In lung, two significantly down-regulated and two up-regulated genes were found (Table 2) (Figure 3b). The magnitude of the

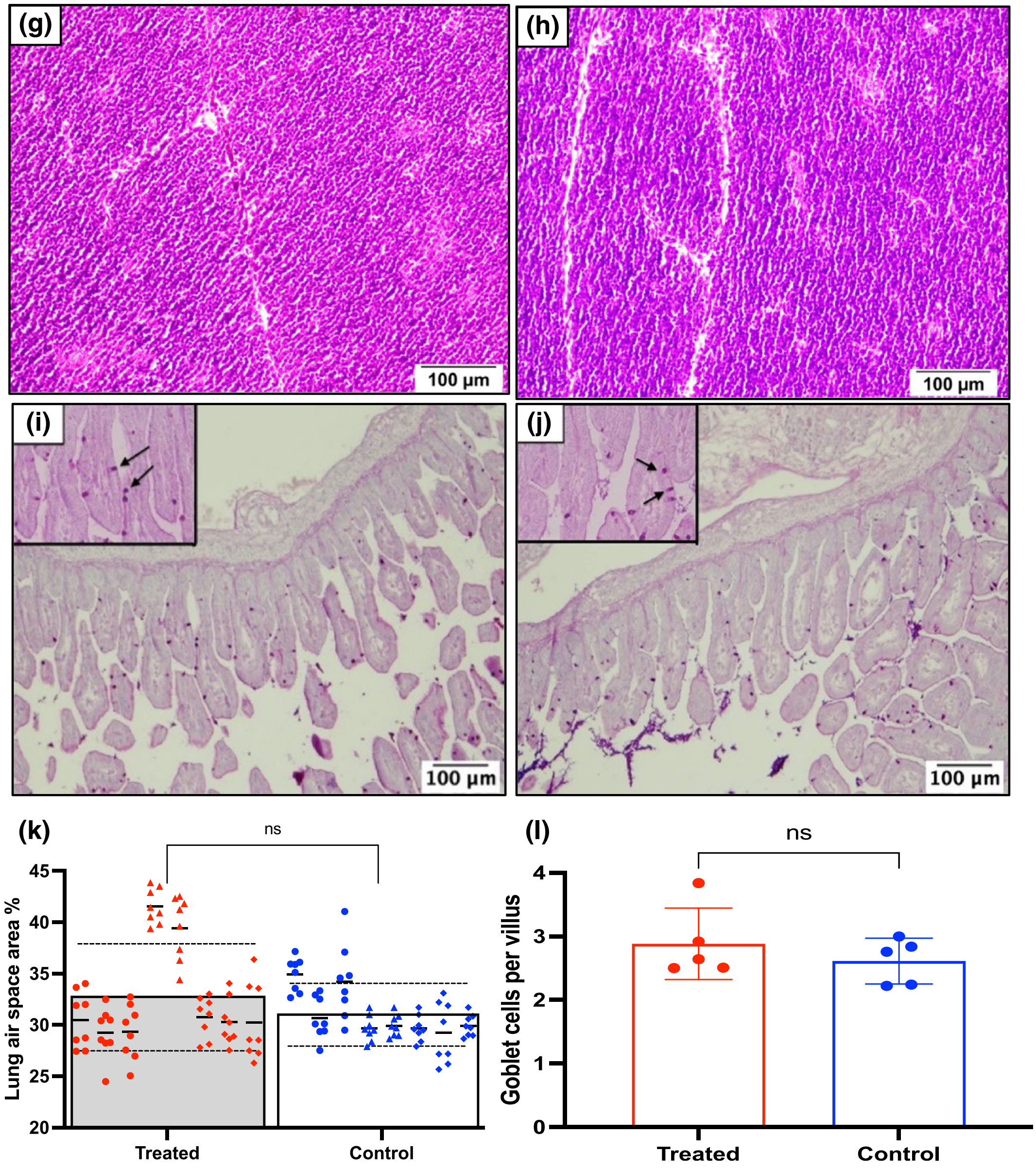

FIGURE 1 (Continued)

expression changes was moderately small (approximately threefold to fourfold change), with the exception that Apob expression was up-regulated by approximately 50-fold in the ETI-treated group. No enriched GO terms or functional protein associations were identified.

3.2.3 | Small intestine

In small intestine, we identified five DEG (one up-regulated and four down-regulated) (Table 2) (Figure 3c). The magnitude of the changes varied from sixfold to over 4000-fold. Protein–protein interactions network analysis did not reveal any enriched GO terms nor any protein–protein association between the DEG.

3.2.4 | Thymus

In thymus, a total of 29 identified DEG were found with 27 up-regulated and two down-regulated (Table 3) (Figure 4a). The top 10 up-regulated genes are listed in Table 3, all of which displayed significantly elevated expression (36-fold to 153-fold) after ETI treatment. Two down-regulated genes displayed small changes (twofold to fourfold). Protein–protein interactions network analysis demonstrated strong association between eight of the top 10 up-regulated genes (Figure 4b). Regarding gene ontology (GO) enrichment analysis, 23 enriched GO terms were identified, composed of 15 biological process and eight cellular component terms (Figure 4c). The top 3 enriched biological process terms were system process, muscle system process and muscle structure development. The three most

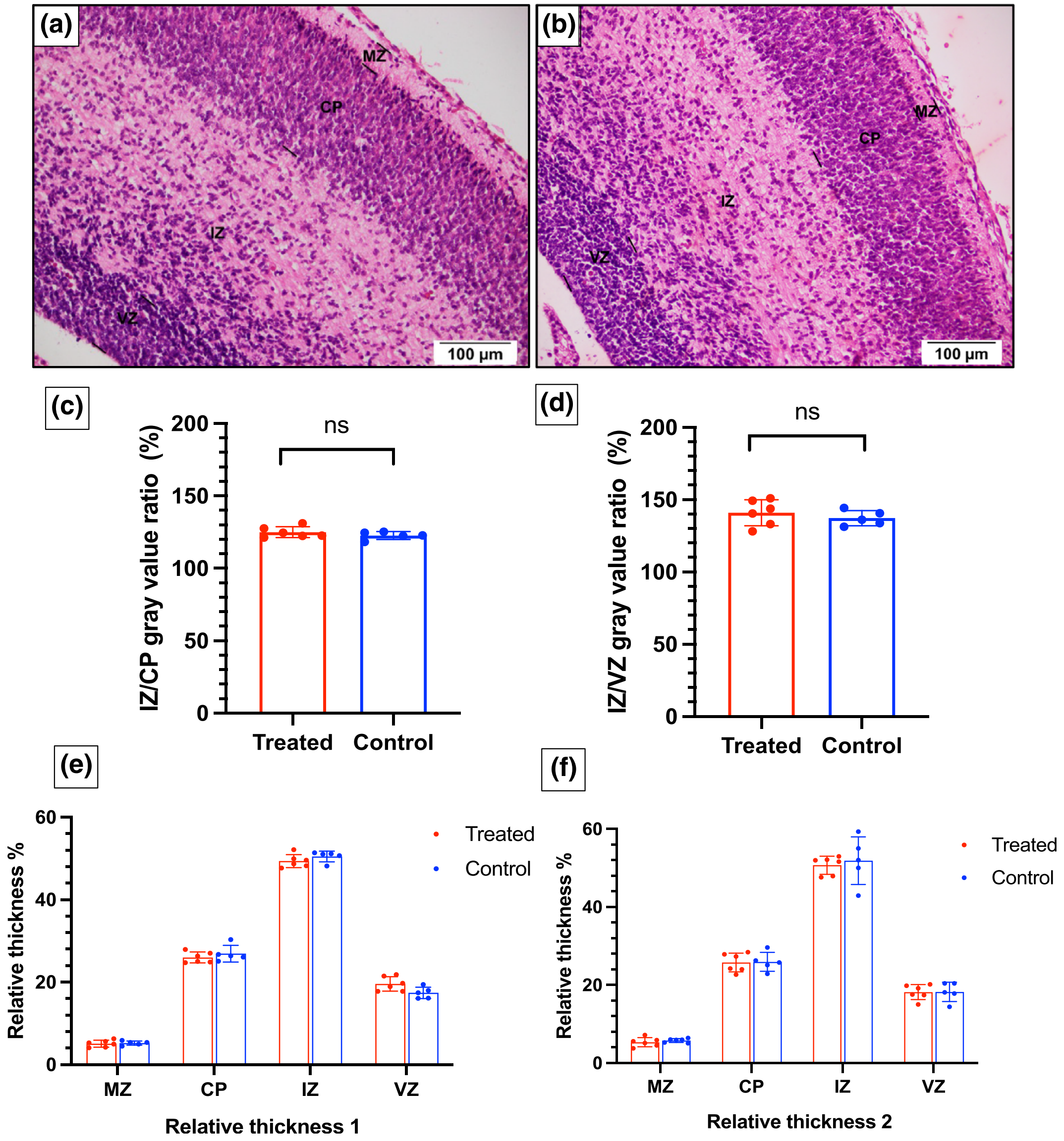

FIGURE 2 Histological analysis of cortex in E19 pups after subchronic elexacaftor/tezacaftor/ivacaftor exposure. (a,b) Representative H&E-stained histological sections of cortex from the ETI-treated (a) and control (b) E19 pups. Scar bar = 100 μm. (c,d) Relative mean grey value in different cortical layers. IZ/CP grey value ratio % = mean grey value in IZ /mean grey value in CP (c); IZ/VZ grey value ratio % = mean grey value in IZ/mean grey value in VZ (d). (e,f) Relative thickness of different cortical layers. The thickness was measured at different locations in each brain (Figure S3). All data were normalized to the thickness of the whole cortex in the same location. The relative thickness in the same location was compared between different pups. Data were analysed by unpaired t test, presented as mean ± SD (c–f). n = 5 (control) or six (treated) pups (pups in each group were from one to two different litters). CP, cortical plate; ETI, elexacaftor/tezacaftor/ivacaftor; E, embryonic day; H&E, haematoxylin & eosin; IZ, intermediate zone; MZ, marginal zone; VZ, ventricular zone.

enriched cellular component terms were supramolecular fibre, supramolecular polymer and supramolecular complex. These significantly enriched GO terms were heavily associated with muscle development.

3.2.5 | Cortex

A total of 48 DEG were identified in cortex, with four up-regulated and 44 down-regulated genes (Figure 5a). The four up-regulated genes revealed moderate increases in expression (twofold to sevenfold). The top 10 down-regulated genes are listed in Table 4 and show large expression reductions (14-fold to 320-fold). Protein–protein interactions network analysis revealed 17 pairs of moderate interactions (Figure 5b). No significantly enriched GO terms were identified, suggesting that these DEG do not coherently represent any specific functions. However, we detected a group of DEG involved in nervous system development (18/48), neurogenesis (14/48), neuron differentiation (13/48) and brain development (11/48).

TABLE 2 Differentially expressed genes (DEG) in liver, lung and small intestine of ETI-treated E19 pups, compared with control pups (ranked by Log2FC). Abbreviation; FDR, false discovery rate.

Liver			
Gene	**Description**	**Log2FC**	**FDR**
Up-regulated			
Asb15	Ankyrin repeat and SOCS box containing 15	2.95	0.046
Gdf15	Growth differentiation factor 15	1.58	0.013
Lung			
Gene	**Description**	**Log2FC**	**FDR**
Up-regulated			
Asb15	Ankyrin repeat and SOCS box containing 15	1.94	0.038
Ccl21	C-C motif chemokine ligand 21	1.49	0.024
Down-regulated			
Apob	Apolipoprotein B	−5.66	0.037
Zmiz2	Zinc finger, MIZ-type containing 2	−1.82	0.00045
Small intestine			
Gene	**Description**	**Log2FC**	**FDR**
Up-regulated			
Tnks2	Tankyrase 2	2.68	<0.0001
Down-regulated			
Cpb1	Carboxypeptidase B1	−22.052	<0.0001
Muc6	Mucin 6, oligomeric mucus/gel-forming	−6.29	0.024
LOC691352	Similar to Robo-1	−6.09	0.00059
Nkx6-3	NK6 homeobox 3	−5.79	<0.0001

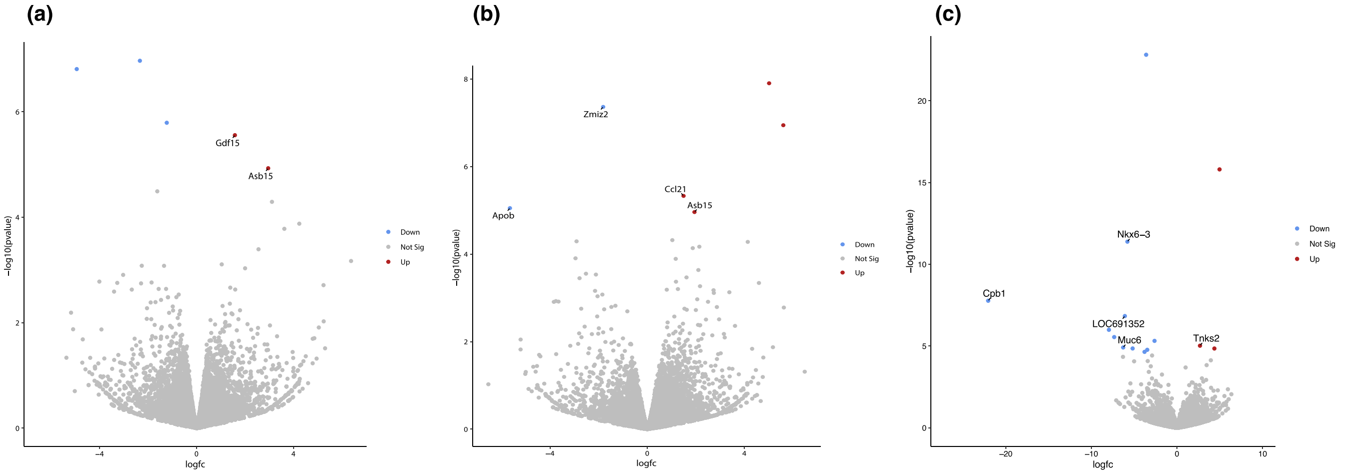

FIGURE 3 Exploratory data: general RNA-seq analysis of liver, lung and small intestine. Volcano plots of DEG in the liver (a), lung (b), small intestine (c) between ETI-treated and untreated control E19 pups. Data were plotted as −log10 (*P* value) versus log 2 fold changes. Dots were represented as significantly up-regulated genes (red), down-regulated genes (blue), and genes that were not significantly changed (grey). Identified DEG were labelled with their gene symbols. n = 3 pups per group (each pup was from a different litter). DEG, differentially expressed gene.

4 | DISCUSSION

Pregnant women with CF require ongoing drug therapy to maintain both maternal and foetal health. Due to the increasing number of women with CF becoming pregnant, patients and clinicians are urgently seeking more safety information for the highly effective drug ETI during pregnancy. Animal reproductive studies and limited case reports suggest ETI may be safe for developing babies, but larger

TABLE 3 Top 10 up-regulated genes and all down-regulated genes in thymus of ETI-treated SD E19 pups, compared with control pups (ranked by Log2FC). Total DEG listed in Table S1. Abbreviation; FDR, false discovery rate.

Gene	Description	Log2FC	FDR
Up-regulated			
LOC298139	Similar to RIKEN cDNA 2310003M01	7.26	0.0087
Myoz1	Myozenin 1	6.84	0.0079
Cacng1	Calcium voltage-gated channel auxiliary subunit gamma 1	6.77	0.0031
Pgam2	Phosphoglycerate mutase 2	5.63	0.013
Prr32	Proline rich 32	5.38	0.0077
Myh1	Myosin heavy chain 1	5.31	0.025
Myl1	Myosin, light chain 1	5.29	0.038
Smtnl1	Smoothelin-like 1	5.24	0.013
Ckm	Creatine kinase, M-type	5.20	0.050
Acta1	Actin, alpha 1, skeletal muscle	5.15	0.037
Down-regulated			
Cnn1	Calponin 1	−2.09	0.013
Camk2g	Calcium/calmodulin-dependent protein kinase II gamma	−1.05	0.038

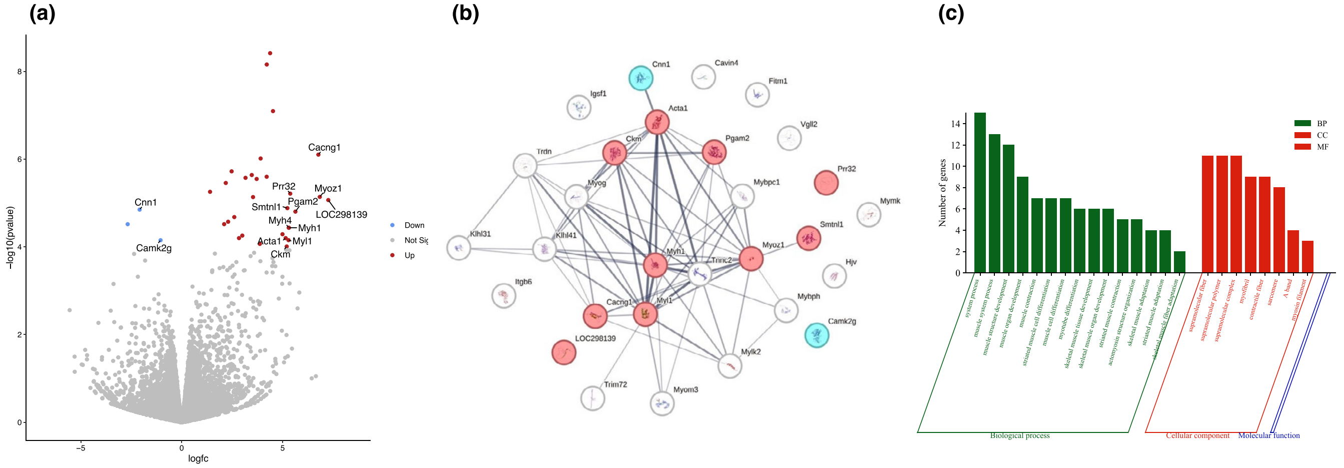

FIGURE 4 Exploratory data: RNA-seq analysis of E19 foetal rat thymus. (a) Volcano plots of DEG in thymus of ETI-treated E19 pups, compared with untreated control E19 pups. Data are plotted as −log10 (*P* value) versus log 2 fold changes on the x-axis. Dots were represented as significantly up-regulated genes (red), down-regulated genes (blue), and genes that were not significantly changed (grey). Top 10 up-regulated and all down-regulated genes were labelled with their gene symbols. (b) Protein–protein interactions (PPI) network analysis of DEG in thymus. Each node represents a gene-encoded protein. Lines between proteins indicate an association and the thickness of the line (if available) indicates the strength of the data supporting their functional association. Red nodes represent top 10 up-regulated genes. Blue nodes represent all down-regulated genes. Networks are built on medium confidence interactions (>0.400 as determined by STRING database). (c) Gene ontology enrichment analysis of thymus. Green, red and blue bars represent the enrichment of DEG in biological process, cellular component and molecular function, respectively. Gene counts in y-axis represent the number of DEG involved in corresponding enriched GO terms. n = 3 pups per group (each pup was from a different litter). DEG, differentially expressed gene; GO, gene ontology; PPI, protein–protein interaction.

cohort studies are simply not available yet. Therefore, in this study, we used a preclinical model to explore the impacts of maternally administered ETI on prenatal offspring.

The extensive accumulation of maternally administered ETI in foetal tissues leads to concern that these CFTR modulators could affect the normal development of babies. Consequently, we first conducted detailed histological assessments of foetal organs in a non-CF pregnant rat model. In foetal pancreas, the E12 to E19 treatment period spans the secondary and tertiary transition stages during which considerable pancreatic cell proliferation, differentiation and structural development occurs (Murtaugh, 2007). In this study, the absence of any structural abnormalities in treated pups suggested CFTR modulators may not affect foetal acini branching morphogenesis (Figure 1a,b). Our data also did not reveal any visible structural damage to foetal liver indicating that hepatocyte proliferation and maturation were not affected by ETI (Figure 1c,d). In the foetal lungs,

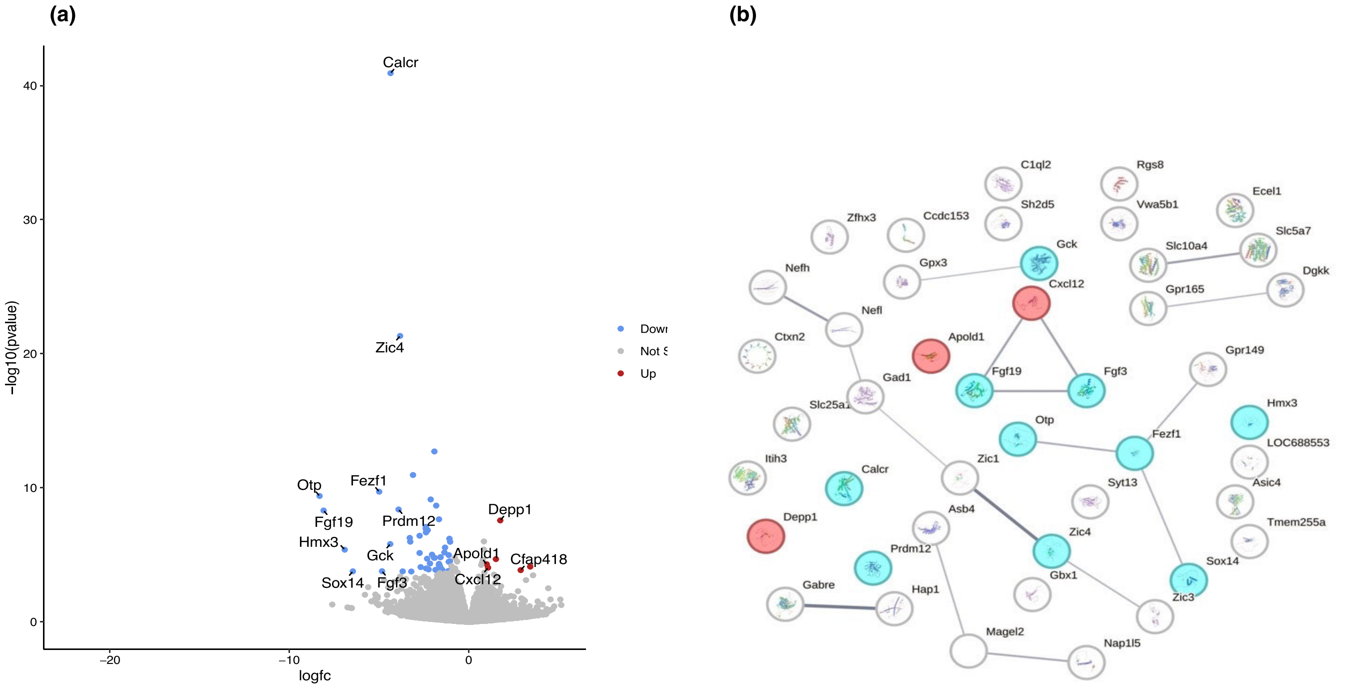

FIGURE 5 Exploratory data: RNA-seq analysis of E19 foetal rat cortex. (a) Volcano plots of DEG in cortex of ETI-treated E19 pups, compared with untreated control E19 pups. Data were plotted as -log10 (*P* value) versus log 2 fold changes. Dots were represented as significantly up-regulated genes (red), down-regulated genes (blue) and genes that were not significantly changed (grey). All up-regulated and top 10 down-regulated genes were labelled with their gene symbols. (b) Protein–protein interactions (PPI) network analysis of DEG in thymus. Each node represents a gene-encoded protein. Lines between proteins indicate an association and the thickness of the line (if available) indicates the strength of the data supporting their functional association. Red nodes represent all up-regulated genes. Blue nodes represent top 10 down-regulated genes. Networks are built on medium confidence interactions (>0.400 as determined by STRING database). n = 3 pups per group (each pup was from a different litter). DEG, differentially expressed gene; GO, gene ontology; PPI, protein–protein interaction.

there was no effect of ETI on the average lung air space in the treated pups compared with controls (Figure 1e,f). The E12 to E19 period mainly overlaps with the pseudoglandular (times of branching morphogenesis) and canalicular (times in which the respiratory tree expands in length and diameter) stages (Li et al., 2023; Lin Liu & Hinsdale, 2014; Schittny, 2017; Warburton et al., 2010). Hence, our findings suggest that ETI at these stages does not directly disrupt epithelial tubule branching and formation of the respiratory tree. This is different from findings from Lhuillier's group, which reports that acute exposure to ETI at the pseudoglandular stage decreases lung branching and increases abnormal terminal dilations, indicating potential negatively impacts of WT CFTR activation and/or off-target effects of CFTR modulators on lung development (Lhuillier et al., 2022). The absence of morphological lung changes in our study may be due to different drug concentrations, treatment method and study models used. Subchronic oral treatment to rat mothers causes significantly lower concentrations of drug to reach foetuses, not causing significant complications, but compared with the extreme *in vitro* mouse embryonic lung culture model used in Lhuillier's study, this *in vivo* model would more closely resemble real clinical cases. Also, compared with mouse, rats express higher level of CFTR mRNA in lungs resembling that of human, making it more representative of CFTR-related study (McCarron et al., 2020; Trezise et al., 1993). Hence, our study suggesting the lack of lung structure damage is more representative of the real clinical response. Similar alveolar air space ratios between treated and control pups also suggests no signs of inflammation nor any wall thickening due to chronic ETI exposure. The structure of foetal thymus was not affected by in utero ETI exposure. The expression of CFTR in thymus is less clear. However, thymus is the site of T cell maturation and T cell function appears to be abnormal in patients with CF, so its normal development after modulator treatment could be a positive sign to maintain the normal T cell-related immune response, which may be beneficial for babies with CF (Tiringer et al., 2013). In jejunum, the number of goblet cells on villi was not significantly affected by the ETI treatment and the shape of goblet cells also appeared normal (Figure 1g,h). Intestinal goblet cells are responsible for mucus production as well as the maintenance of healthy microbial environment (Yang & Yu, 2021). Although the goblet cell differentiation mechanism in rats remains unclear, histological results from this study indicate that ETI does not disrupt intestinal goblet cell development, hence mucus secretion and microecological balance are likely to be maintained. In foetal cortex, the similar relative mean grey value suggested no loss of cells in cortical layers. The comparable relative thickness indicated no swelling or shrinking of cortical layers. CFTR is expressed in neurons of most cortical layers, so our histological results with no significant abnormalities could suggest that CFTR modulators may not affect the development of neurons in CNS (Marcorelles et al., 2014). Overall, despite placentally transferred ETI entering

TABLE 4 Top 10 down-regulated genes and all up-regulated genes in cortex of ETI-treated SD E19 pups, compared with control pups (ranked by absolute value of Log2FC). Total DEG listed in Table S2. Abbreviation; FDR, false discovery rate.

Gene	Description	Log2FC	FDR
Up-regulated			
Cfap418	Cilia and flagella associated protein 418	2.89	0.038
Depp1	DEPP1, autophagy regulator	1.75	<0.0001
Cxcl12	C-X-C motif chemokine ligand 12	1.07	0.029
Apold1	Apolipoprotein L domain containing 1	1.00	0.018
Down-regulated			
Otp	Orthopaedia homeobox	−8.32	<0.0001
Fgf19	Fibroblast growth factor 19	−8.09	<0.0001
Hmx3	H6 family homeobox 3	−6.91	0.0027
Sox14	SRY-box transcription factor 14	−6.46	0.044
Fezf1	Fez family zinc finger 1	−4.99	<0.0001
Fgf3	Fibroblast growth factor 3	−4.83	0.044
Gck	Glucokinase	−4.38	0.0011
Calcr	Calcitonin receptor	−4.36	<0.0001
Prdm12	PR/SET domain 12	−3.91	<0.0001
Zic4	Zic family member 4	−3.83	<0.0001

different foetal tissues extensively, histological results in this study are encouraging in that they provide some initial safety data suggesting a low risk of ETI to foetuses.

Despite the absence of evidence of significant structural abnormalities, it is possible that ETI exposure during foetal development could induce gene expression changes that may not be apparent at a structural or functional level until much later in life. Accordingly, we also compared gene expression patterns (transcriptomes) in several organs between ETI-treated and untreated control E19 pups to identify any DEG.

In both foetal liver and lung, exposure to maternally administered ETI during the E12–E19 period of development resulted in minor changes in gene expression. One notable gene is *Asb15*, which was up-regulated in both tissues after ETI exposure. *Asb15* encodes for a member of the suppressor of cytokine signalling box superfamily and previous studies indicated that this *Asb* gene family is involved in cell proliferation and differentiation (Kohroki et al., 2001; Liu et al., 2003; Xing et al., 2020). Interestingly, CFTR has also been reported to be associated with epithelial proliferation and differentiation (Amaral et al., 2020). It's up-regulation in both organs reinforces the possibility that the enhanced *Asb15* expression could have a yet unknown association with CFTR modulated by ETI. The absence of identified gene down-regulation in ETI-exposed lung also contrasts with Lhuillier's results, consistent with our previous morphological findings that lung branching and terminal buds formation are not negatively affected by small drug concentrations (Lhuillier et al., 2022). In small intestine, the number of gene expression changes was small and these were in mostly uncharacterized genes. The significantly down-regulated gene *Muc6*, encoding mucin6, is one of the main gel-forming mucins secreted by intestinal goblet cells (Pelaseyed et al., 2014). Studies have shown that CFTR is integrated with mucin formation and that ETI treatment reduces mucin secretion and concentration in CF cells (Morrison et al., 2022; Okuda et al., 2022). The down-regulation of the *Muc6* gene found in this study could also be caused by the potential impacts of ETI on intestinal CFTR. Collectively, no significantly enriched GO terms nor any known functional associations were identified between DEG, suggesting that these effects of ETI on gene expression in these tissues are more likely to be unrelated and not part of known developmental pathways. Nevertheless, the impacts on cell proliferation and differentiation, and the intestinal mucus secreting processes, do warrant further investigation, particularly over longer periods of gestational ETI exposure.

In foetal thymus, we identified 29 DEG, 27 of which were up-regulated. Within the top 10 up-regulated genes, *Myoz1*, *Myh1*, Myl1 and *Acta1* encode Z-line protein myozenin, myosin heavy chain 1, myosin light chain 1 and alpha-actin, respectively, which are all structural components of striated muscle (Takada et al., 2001). *Pgam2*, *Smtnl1* and *Ckm* encode phosphoglycerate mutase 2, smoothelin-like 1 protein and creatine kinase M, respectively, responsible for metabolism of striated muscle (Fothergill-Gilmore & Watson, 1989; Major et al., 2021; Watchko et al., 1996). *Cacng1* encodes one calcium voltage-gated channel regulating striated muscle contraction (Freise et al., 2000). These eight largely up-regulated genes are all involved in striated muscle process and are also functionally linked to each other (Figure 4b), suggesting that striated muscle is significantly affected in the thymus after ETI exposure. This also corresponds with GO enrichment analysis results, where most of the enriched terms are muscle related. The thymic medulla contains myoid cells that resemble skeletal muscle, are composed of tightly packed myofibrils and express muscle genes including myosin, actin and myozenin (Bornemann & Kirchner, 1998). Evidence indicates that thymic myoid cells act as antigen-presenting cells to trigger autoimmune response and are responsible for autoimmune diseases like myasthenia gravis (Matsumoto et al., 2004). They also play roles in protecting thymocytes from apoptosis and regulating their differentiation (Le Panse & Berrih-Aknin, 2005). We detected up-regulation of multiple muscle-related genes in this study, suggesting possible expansion of myoid cells. However, the physiological consequence of their expansion remains unclear. Based on their identified biological roles, we hypothesize that the overexpression of myoid-related genes may augment the autoimmune response and increase the risks of autoimmune diseases. It may also impact the differentiation and apoptosis of other thymic cells.

In foetal cortex, there were more DEG than any other tissue investigated, including four up-regulated and 44 down-regulated genes. Top 10 down-regulated genes were listed in Table 4. Otp is a brain-specific homeobox transcription factor expressed in CNS that has been suggested to be involved in synchronization of cortical columns (Morales et al., 2022). *Hmx3* is another homeobox gene controlling the embryonic development of CNS (Wang & Lufkin, 2005). *Sox 14* encodes a SOX-family transcription factor regulating neuronal differentiation and cell cycle progression (Katsuyama et al., 2022). *Fezf1* encodes a transcription factor controlling neurogenesis in early development of cerebral cortex (Shimizu et al., 2010). *Fgf3* is a member of fibroblast growth factor family important for cortical patterning and size (Rubenstein, 2011; Turner et al., 2016). *Zic4*, along with other two less down-regulated genes *Zic 3* and *Zic1*, encode zinc-finger proteins. The *Zic* family are involved in a variety of neural development processes including neurogenesis and skeletal patterning (Aruga, 2004; Houtmeyers et al., 2013). *Prdm12* is also a subfamily of Krupple-like zinc finger protein, responsible of neurogenesis and neuronal differentiation (Rienzo et al., 2021). Additional genes involved in neurogenesis and neuron differentiation such as *Nefl*, *Nefh* and *Hap1* were also significantly down-regulated (Fernandez-Martos et al., 2015; Xiang et al., 2014). Collectively, our RNA-seq results suggest that maternally administered ETI may have the potential to affect prenatal brain growth and future central nervous system development and maturation. Foetal rat cortical development in mid to late pregnancy stages is marked by explosive development of neurons and widespread CFTR expression was detected in neurons of almost all the cortical layers (Marcorelles et al., 2014; Rash et al., 2011; Semple et al., 2013). Hence, CFTR modulators entering the developing brain could accumulate around CFTR in neurons of all cortical layers and might affect neurogenesis, neural differentiation and other processes there. Further investigation is required to elucidate the precise neurological effects.

5 | CLINICAL IMPLICATIONS

In our non-CF pregnant rat model, insignificant structural and genetic changes in most of the foetal tissues examined provide some evidence for clinicians that collateral exposure of foetuses to ETI may be low risk. However, the larger numbers of gene changes detected in thymus and cortex suggest some caution as the consequences of these changes are not known and may not manifest until much later in postnatal life. Thus, the effects of ETI on developing thymus and brain should still be considered as a potential risk when considering taking ETI during pregnancy until more information from longer term studies is available.

6 | LIMITATIONS

Our study has limitations. Firstly, non-CF pregnant Sprague–Dawley rats were mainly focused on all experiments, as most pregnant women with CF give birth to babies that are healthy carriers and do not develop CF pathologies. Future research using pregnant F508del-CFTR transgenic rats is required to assess the action of ETI on mothers with CF and resulted foetuses. Secondly, study with larger sample size is required to avoid potential underestimation of ETI impacts on structure and gene expressions of offspring. Thirdly, we only treated the pregnant rats during the last week of gestation, equivalent to approximately 21 to 33 weeks of human gestation which overlaps with the second and third trimester (Agoston, 2017). The effects of maternally transferred ETI on earlier gestational stages, which includes crucial organogenesis and morphogenesis, remains to be explored in follow up long term studies. Furthermore, given the unexpected findings of the prominent gene changes in foetal thymus and cortex, we are in progress of conducting further proteomics study for validation of key gene mutations in these two organs.

7 | CONCLUSION

In many foetal tissues, 7 days of subchronic exposure to ETI did not result in any significant histological or genetic changes, suggesting ETI may not pose a major safety risk for healthy babies. These observations may be helpful for clinicians and mothers with CF deciding whether to remain on ETI therapy during pregnancy and breastfeeding. However, we identified that thymic myoid cells and brain neural development may be affected by ETI treatment due to a greater number of gene expression changes found in the thymus and brain. The functional significance of these gene changes is unknown and warrants further longer-term studies to determine the safety of ETI on brain and thymus development.

AUTHOR CONTRIBUTIONS

D. Li: Data curation (lead); formal analysis (equal); visualization (equal). **M. Donnelley:** Formal analysis (supporting); funding acquisition (supporting); writing—review and editing (supporting). **D. Parsons:** Methodology (supporting); writing—review and editing (supporting). **M. Habgood:** Data curation (supporting); formal analysis (supporting); methodology (supporting); validation (supporting); writing—review and editing (supporting). **E. Schneider-Futschik:** Conceptualization (lead); data curation (supporting); formal analysis (supporting); funding acquisition (lead); investigation (equal); methodology (equal); project administration (equal); resources (lead); supervision (lead); validation (lead); visualization (supporting); writing—original draft (equal); writing—review and editing (lead).

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ACKNOWLEDGEMENTS

Open access publishing facilitated by The University of Melbourne, as part of the Wiley - The University of Melbourne agreement via the Council of Australian University Librarians.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

ORCID

Elena K. Schneider-Futschik https://orcid.org/0000-0001-6044-4824

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How to cite this article: Li, D., Donnelley, M., Parsons, D., Habgood, M. D., & Schneider-Futschik, E. K. (2024). Extent of foetal exposure to maternal elexacaftor/tezacaftor/ivacaftor during pregnancy. *British Journal of Pharmacology*, *181*(15), 2413–2428. https://doi.org/10.1111/bph.16417