Journal of Cystic Fibrosis 25 (2026) 13–20

Contents lists available at ScienceDirect

Journal of Cystic Fibrosis

journal homepage: www.elsevier.com/locate/jcf

Original Article

Exposure of dams to Elexacaftor/ Tezacaftor/Ivacaftor during pregnancy and breastfeeding induces reversible alterations in newborn wild type CD-1 mice

Angelica Squarzoni [a], Gaia Boschetti [a], Sine Mandrup Bertozzi [a], Maria Summa [b], Angelo Serani [c], Elisa Milandri [d], Roberto Mandrioli [e], Michele Protti [d], Laura Mercolini [d], Caterina Montani [f], Giovanna Capodivento [f], Giuliana Cangemi [g], Nicoletta Pedemonte [g], Tiziano Bandiera [h], Fabio Benfenati [f,i], Lucilla Nobbio [f], Rosalia Bertorelli [b], Andrea Armirotti [a,*]

[a] *Analytical Chemistry Facility, Istituto Italiano di Tecnologia, Via Morego 30, 16163, Genova, Italy*
[b] *Translational Pharmacology Facility, Istituto Italiano di Tecnologia, Via Morego 30, 16163, Genova, Italy*
[c] *Genetics and Epigenetics of Behavior, Istituto Italiano di Tecnologia, Via Morego 30, 16163, Genova, Italy*
[d] *Research Group of Pharmaco-Toxicological Analysis (PTA Lab), Department of Pharmacy and Biotechnology (FaBiT), Alma Mater Studiorum - University of Bologna, Via Belmeloro 6, 40126, Bologna, Italy*
[e] *Department for Life Quality Studies (QuVi), Alma Mater Studiorum - University of Bologna, Corso D'Augusto 237, 47921 Rimini, Italy*
[f] *IRCCS Ospedale Policlinico San Martino, Largo Rosanna Benzi 10, 16132, Genova, Italy*
[g] *IRCCS Istituto Giannina Gaslini, Genova, Italy*
[h] *D3 PharmaChemistry, Istituto Italiano di Tecnologia, Via Morego 30, 16163, Genova, Italy*
[i] *Center for Synaptic Neuroscience and Technology, Istituto Italiano di Tecnologia, Largo Rosanna Benzi 10, 16132, Genova, Italy*

ARTICLE INFO

Keywords:
Cystic fibrosis
ETI
Tezacaftor
Dihydroceramides
Myelin

ABSTRACT

Background: We previously demonstrated that Tezacaftor inhibits the enzyme (DEGS) that converts dihydroceramides (dHCer) into ceramides, thus producing accumulation of dHCer in various cells and tissues. We here conducted an *in-vivo* safety study, by administering ETI to CD-1 mice during pregnancy and breastfeeding.
Methods: ETI was incorporated into mouse food (in a high-fat diet regimen). Pups' behavior was measured with SHIRPA tests. ETI and dHCer levels in plasma and tissues, as well as changes in the global lipidome were measured by tandem mass spectrometry coupled to liquid chromatography.
Results: At 10 days after birth, we observed a significant accumulation of dHCer in the brains of pups born from ETI-fed dams compared to controls. No accumulation was observed in the sciatic nerve of these animals, likely due to much lower levels of ETI compared to the brain. We also conducted an untargeted lipidomics survey, which revealed other alterations in lipid metabolism associated with exposure to ETI during pregnancy. During breastfeeding, given the negligible exposure to the drug, these alterations revert and virtually disappear at P28, together with other differences in the phenotype and behavior of the pups observed earlier during development.
Conclusions: We here demonstrate that exposure to ETI during pregnancy is associated with observable molecular changes in the brain lipidome, which are not likely limited to the inhibition of DEGS. These changes are reverted when exposure to ETI ceases.

1. Introduction

The triple drug combination Elexacaftor/Tezacaftor/Ivacaftor (ETI) represents a powerful therapeutic option for people with cystic fibrosis (pwCF) eligible to receive this treatment. Its introduction in clinical practice (approved by FDA in 2019 and by EMA in 2020), has positively affected the quality of life of thousands of pwCF worldwide. On the other hand, several side effects, mostly neuropsychological, have been reported for this drug [1–4]. Our group recently demonstrated [5,6] that Tezacaftor is able to induce accumulation of dihydroceramides (dHCer)

* Corresponding author.
E-mail address: andrea.armirotti@iit.it (A. Armirotti).

https://doi.org/10.1016/j.jcf.2025.11.004
Received 9 September 2025; Received in revised form 6 November 2025; Accepted 6 November 2025
Available online 14 November 2025
1569-1993/© 2025 The Authors. Published by Elsevier B.V. on behalf of European Cystic Fibrosis Society. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

in different cells by inhibiting the delta-4 sphingolipid desaturase (DEGS) enzyme, which converts dHCer into ceramides (Cer) along the de novo synthetic pathway of sphingolipids [7,8]. By investigating this effect on primary human bronchial epithelial (HBE) cells from both wild-type (WT) and homozygous F508del-CFTR individuals, and from subjects carrying two CFTR minimal function, non-rescuable, mutations, we also demonstrated that the effect is genotype independent [5] and it is thus an off-target effect of the drug. This evidence might be of relevance from the drug safety standpoint: based on the information included in the original EMA and FDA [9,10] drug reports, as well as in current literature [11], Tezacaftor can 1) cross both the placental and the blood-brain barriers and 2) pass into the mother's milk. It can thus be concluded that newborns from mothers undergoing ETI therapy are exposed to Tezacaftor from the very beginning of the pregnancy. On the other hand, defective DEGS is linked to abnormal development of the central (CNS) and peripheral nervous systems (PNS), with altered myelin formation and maintenance [12–15]. Moreover, individuals with defective DEGS frequently develop epilepsy [13,14]. Myelin is a unique lipid-rich membrane structure with sphingolipids being critical components [16–20]. Continuous DEGS inhibition, by inducing an altered balance between sphingolipids and their dihydro- counterparts, might cause alterations in the development of the CNS and PNS, with a potential onset of altered phenotypes later in life. DEGS is also involved in the mechanism of phototransduction by retinal photoreceptors [21] and thus potential harmful effects of prolonged enzyme inhibition on vision cannot be totally ruled out. To address these potential safety concerns, we have designed an animal study mimicking, in WT CD-1 mice, the exposure to ETI occurring during human pregnancy, breastfeeding and development, by administering ETI in food to dams, before and throughout the pregnancy and for the first month after mice's birth. Fig. 1 depicts the general scheme of the experiment.

With this experiment, we aimed at dissecting potential side effects of dHCer accumulation occurring during the critical phases of nervous system development. Other studies demonstrated that this time window is crucial for ETI exposure [22]. We here present the results of a set of molecular, phenotypical and behavioral analyses conducted on pups and aimed at detecting possible dHCer accumulation, as well as other alterations in tissues.

2. Materials and methods

2.1. Solvents and chemicals

Sphingolipid standards were purchased from Avanti Polar Lipids (Alabaster, Alabama USA). Solvents and chemicals were purchased from Sigma-Aldrich (Milan, Italy).

2.2. Food production and incorporation

The high-fat diet (HFD) pellets (Mucedola, Italy), a diet with adjusted calories from proteins 23 %, from fat 42 %, from carbohydrates 45 %, was purchased as control diet. Elexacaftor, Ivacaftor and Tezacaftor (purchased from Twin Helix) were incorporated by the food producer in HFD as powder, at a concentration of 25 mg/Kg, 165 mg/Kg and 38 mg/Kg, respectively, assuming a total of 7-9 gr of food consumption per mice per day. Pellets for both diet regimens (HFD and HFD+ETI) were maintained at 4°C during the whole experiment.

2.3. In vivo experiments

In vivo experiments were performed in wild type CD-1 mice, as established in the guidelines approved by the European Union Council's Directive (Directive 2010/63/EU of 22 September 2010) and by the National Council on Animal Care of the Italian Ministry of Health. The study was authorized by the Italian Ministry of Health with Authorization: 1067/2024. CD-1 female and male mice (Charles-Rivers, Calco, Italy) were kept under a 12-hour light/dark cycle with lights on at 8:00 am, relative humidity of 55 ± 10 %, and at a controlled temperature of 21 ± 1°C. A total of four CD-1 females were designated to each diet group. CD-1 females were fed to HFD or HFD+ETI for three weeks and then mated with CD-1 male mice. After mating, the HFD or HFD+ETI treatment continued during the whole pregnancy and breastfeeding, until sacrifice. The food and water intake were daily checked to verify ETI exposure. The dam's weight was verified weekly. For two dams/group, the litter was sacrificed 10 after birth. The other two litters/group were sacrificed 28 days after birth. In both P10 and P28 pups and dams, brains spinal cord and blood samples were collected. The tissues were frozen in dry ice. From the dams' blood, plasma was obtained by centrifugation (3500 rpm for 15 minutes at 4°C). Blood from pups was collected through VAMS. All the samples collected were stored at –80°C before molecular analysis. For P10, 18 and 15 animals were analyzed for

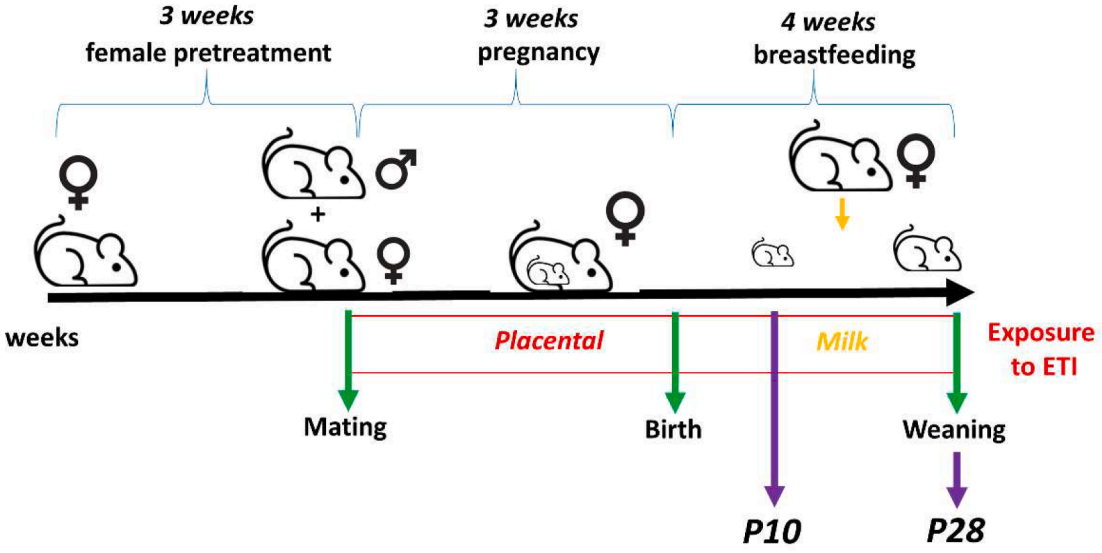

Fig. 1. General scheme of the *in vivo* experiment of ETI exposure during pregnancy and breastfeeding. The results reported in the present study refer to pups sacrificed 10 and 28 days after birth (P10 and P28).

the ETI and control arm respectively. For P28, 10 individual pups were analized for both ETI and control group.

2.4. Sample preparation, dHCer and global lipidomics analysis by LC-MS/MS

Brains were homogenized using IKA T10 basic ULTRA TURRAX homogenizer from IKA-Werke (Staufen, Germany). Samples were homogenized in isopropanol (IPA) using a ratio of 1 mL solvent per 100 mg tissue. Given their tiny weight, sciatic nerves were not homogenized but simply sonicated in IPA: chloroform (8:2) using a ratio of 1 mL solvent per 8 mg tissue. Samples were briefly vortexed, sonicated in ice for 10 minutes and then centrifuged at 9000 g for 30 minutes at 4°C using an Allegra X-14R Refrigerated Centrifuge from Beckman Coulter (Brea, USA). Subsequently, the collected supernatants were dried under nitrogen flux for 4 hours. At the time of analysis, the samples were reconstituted in methanol: chloroform (9:1). Quality control samples (QCs) were produced pooling together aliquots of brain or sciatic nerve, respectively generated. Quantification of dHCer was performed as already described [6]. In short, all samples were randomized along with their QCs and analyzed by UHPLC Shimadzu ExionLC AE liquid chromatography system coupled with quadrupole-trap 7500+ mass spectrometer, purchased from Sciex (Framingham, USA). Separations of dHCer were performed on a reverse CSH C18 column (Waters (Milford, USA), 1.7 µm particle size; 2.1 × 50 mm internal diameter and length). Mobile phase A and B consisted of acetonitrile/water (60:40, v/v) and isopropanol/acetonitrile (90:10, v/v), both added with 10 mM ammonium formate. The column temperature was set at 55°C, the mobile phase flow rate to 0.45 mL/min and injection volume was set to 2 µL for dHCer quantification in brain. An 8-minute gradient was employed for the relative quantification of dihydroceramides (**Table S1**). For mass spec detection, the curtain gas was set to 40 psi, source temperature to 450°C and spray voltage to 1800 V; dHCer were quantified by multiple reaction monitoring (MRM). Table S1 reports all the LC gradients and MRM parameters used for the MS/MS quantification of dHCer and for the global lipidomics profiling.

2.5. Sample preparation and ETI quantification in fluids and tissues by LC-MS/MS

ETI was quantified in fluids and tissues using the method already described [6]. Tezacaftor metabolite M1 was added to the quantification method by optimizing the corresponding MRM detection parameters using an authentic standard kindly provided by a collaborator. The dams' plasma was extracted with acetonitrile in a 1:3 vol/vol ratio. After centrifugation (15 minutes at 9000 g), an aliquot of the surnatant was diluted 1:1 with water + 0.1 % formic acid and analyzed by LC-MS/MS. Exactly 10 µl of pups blood was sampled using VAMS Mitra devices (Neoterix). ETI compounds were extracted from the VAMS tips using 200 µl of methanol, sonicated and then centrifuged at 8000 g (15 minutes each step). The resulting solution was then dried under nitrogen. On the day of analysis, samples were first redissolved in 100 µl of water/acetonitrile 50/50 (both added with 0.1 % formic acid) then analyzed. ETI was quantified in brains from the same vial used for dHCer analysis. Quantification was done against a calibration of authentic standards of ETI compounds in matrix.

2.6. SHIRPA test and data analysis

In each group, the pups' wellbeing was evaluated every day from birth to P28. The first set of two dams/group was sacrificed, along with their litter, 10 days after pups' birth. We decided to evaluate first the possible accumulation of dHCer and ETI exposure in P10 pups and, when we confirmed it, we went on with other two dams/group to evaluate dHCer accumulation and ETI exposure at the end of pups' younghood (P28). During this second experiment, we evaluated pups' behavior and general development through the SHIRPA test at P14, P21 and P28 timepoints as described in the next section. The evaluation of sensory and motor abilities of pups during early development was performed through the SHIRPA (SmithKline Beecham, Harwell, Imperial College and Royal London Hospital Phenotype Assessment) test at postnatal days 14, 21 and 28 (P14, P21, P28). The SHIRPA test performed was the one adapted to test mice pups, previously described by Naskar et al.[23] . The SHIRPA test was performed during the morning, in a controlled environment. The test was performed maintaining the same task order for each pup. The number of animals tested for the HFD treated group was 20. For the HFD+ETI treated group, the number of animals tested was 24. The tasks were divided into four categories: "primary", "sensory", "motor" and "others". The primary tasks were eye reflexes, toe pinch and suckling reflex. The sensory tasks were body position, spontaneous activity, transfer arousal, positional passivity for tail, neck and supine position, whisker reflex, limb grasping and ear reflexes. The motor tasks were trunk curl, grip strength, wire maneuver, contact righting reflex, negative geotaxis and cliff avoidance. The tasks locomotor activity, touch escape, palpebral closure and snout reflex were categorized as "others". The locomotor activity was tested for 2 and 5 minutes in an arena, obtaining information about the walking distance and the rear number for each pup. The body position task was performed in a transparent jar and the rear's number was counted. Table S2 reports the details of SHIRPA scoring we used for our experiments. Primary reflexes, motor functions, or sensory functions indices were calculated as sum of the individual test score as reported in Table S2. A two-way ANOVA was used to determine if there were differences in the primary reflexes, motor functions, or sensory functions indices between the treatments over postnatal days. Tukey's HSD test was used for post-hoc comparisons.

2.7. Statistical analysis

For univariate data analysis of dHCer, as well as for mice weight and length, Excel (Microsoft) and Prism v8 (GraphPad, U.K.) were used. Data from the global lipidomics profiling were analyzed with MetaboAnalyst [24] (Canada). Missing values were replaced by 1/5 of the minimum value observed for that given feature and lipids showing a CV % higher than 25 % in the QC samples were excluded from the dataset. All data were then normalized by the sum of all observed lipid LC-MS/MS peak areas, Log10 transformed and then autoscaled (mean-centered and divided by the standard deviation of each variable). For the volcano plot analysis, a p-value lower than 0.05 (after correction for multiple testing) and a fold change between -1.5 and +1.5 were selected as thresholds.

3. Results and discussion

3.1. Selection of the ETI dose

Given its poor solubility, the administration of ETI through food was the only viable option for a long-term study. To this purpose, to better mimic the high-fat diet (HFD) regimen recommended for pwCF to favor the absorption of ETI, we incorporated ETI into a HFD specifically designed for mice. ETI was incorporated by the food producer as powder. The control arm was fed with the HFD formulation, not fortified with ETI. The amount of ETI to incorporate in the food was carefully assessed, with the aim of choosing a dose able to mimic the real-life scenario of human exposure to ETI during pregnancy and breastfeeding. Indeed, most of the papers currently available on this topic report the administration of ETI at a dose roughly equivalent to the human dose, as summarized by Schneider-Futschik and co-workers [22,25] for data collected in rats. For our experiment, we made a different choice. Based on current recommendations for the selection of the dose for pharmacological studies between different species (*A simple practice guide for dose conversion between animals and human* [26]), we applied allometric scaling to *convert* the human dose to mice. As recommended,

we thus converted the human dose by applying the correction factor 12.3, which accounts for the different body surface area (0.007 for mice Vs 1.62 for a 60 Kg human). This conversion yields an equivalent dose of 41, 21 and 62 mg/Kg for Elexacaftor, Tezacaftor and Ivacaftor. On the other hand, this choice is in line with currently available data on Tezacaftor, the only compound which has the inhibitory effect on DEGS and thus might induce dHCer accumulation *in vivo* [6]. Plasma concentration of Tezacaftor observed in clinical settings (chronic oral administration) in adult pwCF is in the 2.0 - 4.8 μg/mL range [27]. Based on Vertex Toxicokinetic data for Tezacaftor (FDA-submitted non-Clinical Review for application N: 210491Orig1s000, Table 21, Page 54, publicly available document reference ID 4215948) similar plasma concentrations were found in mice treated at 30 mg/Kg dose orally for three months, a dose even higher than the one chosen by us (21 mg/Kg). We also verified the final amount of each component of ETI incorporated in HFD food and we found it was roughly 75-80 % of the planned dose. This result is overall consistent with a 20 % loss expected by the producer occurring during incorporation. We then pre-exposed two female mice per group (HFD and HFD+ETI) for three weeks before mating. Exposure continued during pregnancy (19-21 days) and breastfeeding (10 days). Ten days after birth (P10), the four dams were sacrificed along with 18 pups for the ETI arm and 15 for the control HFD arm. Dams' plasma and pups' brains and sciatic nerves were collected. Pups' blood was also collected using VAMS devices for microsampling [28–30]. For the P28 experiments, the same scheme (pre-exposure, pregnancy, breastfeeding exposure) was applied to three dams/group. As for P10, plasma, blood and tissues were collected from both dams and pups.

3.2. Tezacaftor levels in dams and pups

We first measured ETI plasma levels in the dams, to assess the real exposure to Tezacaftor during pregnancy and breastfeeding for our model. Table 1 reports the levels observed in our study compared to the levels observed in pwCF (2 studies), in pregnant CF women undergoing ETI treatment and compared to the above-referenced Vertex ToxPK data.

Based on this data, we can conclude that, thanks to the allometric scaling we applied, the exposure to Tezacaftor (calculated as parent + M1 metabolite) is quite comparable with current studies on pwCF undergoing ETI and that our model is thus representing a realistic scenario for exposure to Texacaftor during pregnancy. Indeed, considering the amount of drug lost in the food incorporation step, our study corresponds to a dose around half (17 mg/Kg) of that of the Vertex ToxPK study (30 mg/Kg). The different administration mode (food consumption Vs intraperitoneal injection) likely accounts for the higher Tezacaftor levels observed in the Vertex study. We are thus confident that our study represents a good model to explore the safety of this drug during pregnancy. We also quantified Tezacaftor, the inhibitor of DEGS activity, in the blood and brain of P10 and P28 pups, as shown in Table S3. Consistently with previously reported data [11], Tezacaftor passes the placental barrier and the BBB, but it is poorly abundant in the mother's milk, and exposure to Tezacaftor at P28 was significantly lower than at P10 (29 Vs 58 ng/ml). The sharp P10-P28 decrease in Tezacaftor brain exposure (143 Vs 33 pg/ml) mirrors the transition from placental to breastfeeding exposure to the drug. Indeed, the Tezacaftor levels at P28 would likely be much lower on pure breastfeeding, because the pups, not separated from the dams, begin eating small pieces of food beginning from P23-25 and ETI intake accordingly increases.

Table 1
Tezacaftor levels observed in plasma in our study in mice were compared to Vertex ToxPK data (mice) and compared with two available human datasets, collected from pwCF undergoing ETI. N/A= data not available. *= calculated assuming a body weight of 60 Kg and 100 mg Tezacaftor administered daily.

Dataset	Tezacaftor target dose mg/Kg	Tezacaftor (μg/mL, min-max)		
		Parent	*M1 metabolite*	*Total*
Mice, present study	21 (~17 measured)	0.3-0.7	1.5-2.0	1.8-2.7
Mice, Vertex Tox data, day 177	30	2.1-2.4	4.4-5.7	6.5-8.1
Human (Pigliasco et al.) [27]	1.66*	1.4-4.2	N/A	1.4-4.2
Human (Vonk et al.) [31]	1.66*	1.2-10.7	3.7-7.6	4.9-18.3
Pregnant CF women [32]	1.66*	0.5-9.0	N/A	0.5-9.0

3.3. Levels of dihydroceramides in brain at P10 and P28

At P10 and P28, pups' brains were collected and dHCer were measured, as shown in Fig. 2.

A marked accumulation in the global dHCer levels was observed in the brain at P10. This accumulation reverts at P28. While the data presented here refer to the total dHCer content, all the individual dHCer species we monitored in the brain (16:0, 18:0, 18:1, 20:0, 22:0, 24:0 and 24:1) show similar trends as their sum at both P10 and P28 (Figures S1 and S2), with a roughly 2-3 fold increase in the brain of HFD+ETI group compared to the HFD group at P10. This trend was consistently observed in all individuals. On the other hand, the accumulation of dHCer observed in the brain following ETI exposure is in line with our previously published data [6]. This could have been only hypothesized, but not taken for granted, for an exposure to Tezacaftor occurring *in-utero*. Interestingly, the observed increase in dHCer induced by prolonged DEGS inhibition (roughly 2-3-fold) is quite in line with the increase observed in human individuals with aberrant DEGS genotypes[14], with dihydrosphingolipids reaching 40 % of the total sphingolipidome compared to 10 % observed in control subjects. Conversely, brain dHCer levels return to control levels at P28. This is consistent with the exposure to Tezacaftor that virtually ceases during breastfeeding, as discussed above, thus allowing DEGS (no longer inhibited) to re-equilibrate the saturated/desaturated sphingolipids balance. Overall, we can now conclude that exposure of the female mice to ETI during pregnancy induces a significant accumulation of dHCer in the pups' brains at birth. When exposure to Tezacaftor ceases, DHCer accumulation reverts. As additional data, we measured both Tezacaftor and dHCer in the sciatic nerve of these animals, to understand whether DEGS inhibition is limited to the CNS or it also extends to the PNS. No significant changes in dHCer levels were found in the sciatic nerve of ETI pups, at any time point. Since the levels of Tezacaftor measured in the PNS are roughly 1/20 compared to those observed in the brain (7 Vs 143 pg/mg at P10), this result is not surprising.

3.4. Global lipidomics survey of pups brain

Similarly to what we did a few years ago on HBE [33], on the same pups' brain samples we also conducted a global lipidomic survey at both P10 and P28, by monitoring the levels of 536 individual lipid species, ranging from free fatty acids, phospholipids, cholesteryl esters, to glycerolipids and a broad range of sphingolipids. Dihydroceramides were intentionally excluded from this investigation to focus on other possible altered lipid species. Indeed, our data revealed that dHCer are not the only altered lipids at P10, as shown in Fig. 3.

Based on this lipidomic dataset, at P10, the two experimental groups (ETI and control) are well separated in the Scores plot of a Principal Component Analysis (Fig. 3, Panel A), with the ETI treatment accounting for around 23 % of the overall observed variability of the dataset. A set of 63 individual lipids, reported in Supplementary File 1, are significantly ($p<0.05$, with a fold change of at least 50 %) dysregulated between the two groups, as demonstrated by the corresponding Volcano plot (Fig. 3, Panel B). Among other results, this investigation revealed a

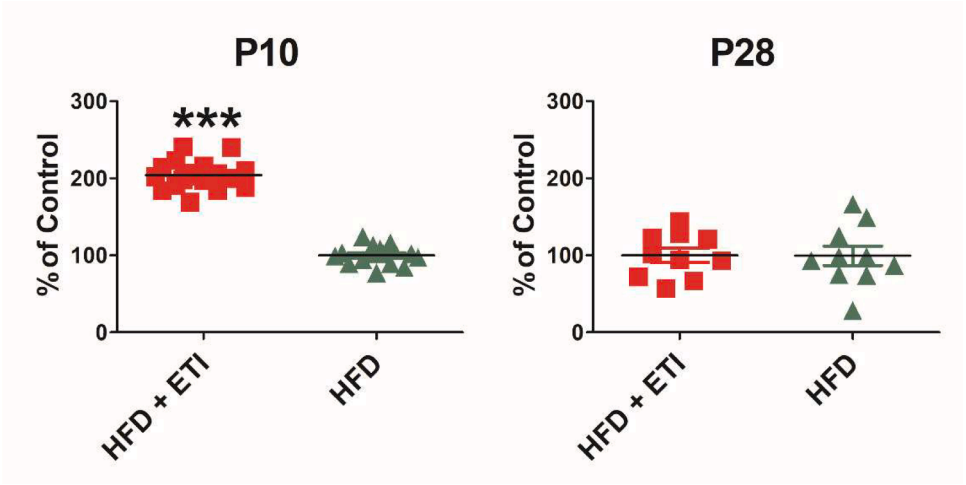

Fig. 2. Brain dihydroceramides levels at P10 (left) and P28 (right) of pups for the ETI arm compared to the control arm. Data (as sum of all dHCer species) are reported as means ± SEM, for $N = 18$ and 15 for ETI and control groups, respectively at P10 and $N = 10$ for both groups at P28 (*** $p < 0.001$, two-tailed *t*-test).

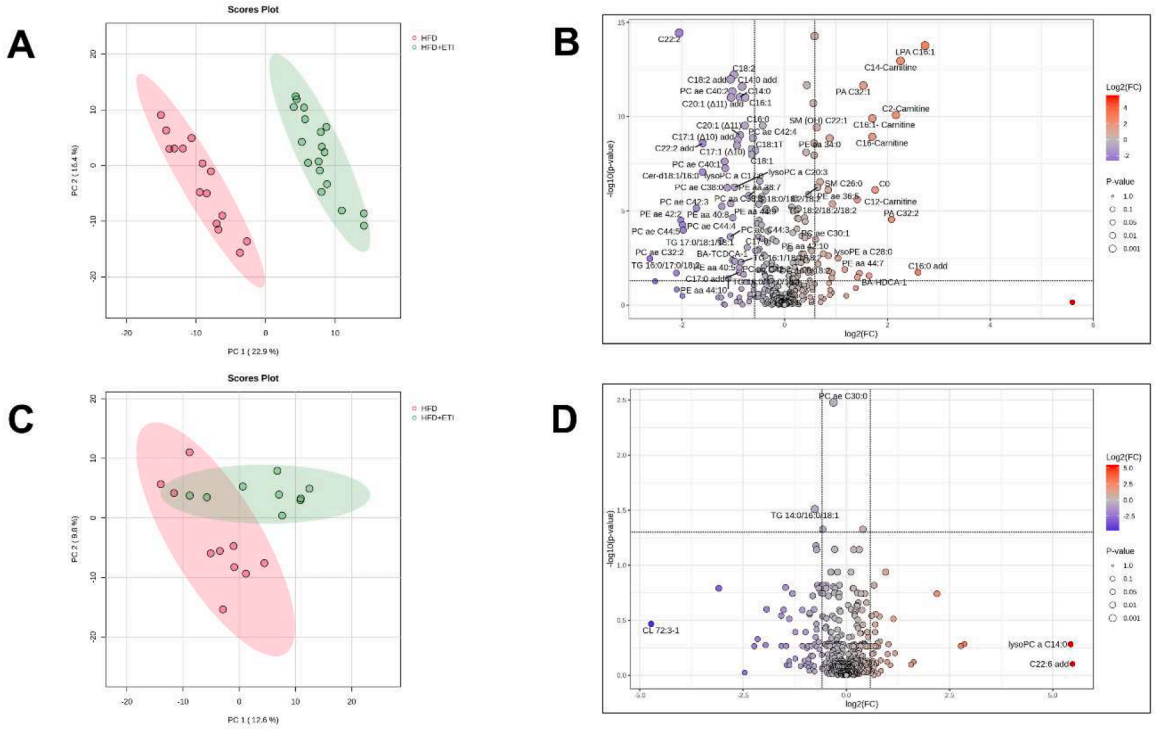

Fig. 3. Results from the global lipidomics profiling of brains of P10 (top) and P28 (bottom) pups born from either ETI or control dams. The Scores plot from the Principal Component Analysis (Panel A) highlights that the two groups have clearly different lipidomic profiles at P10, with 63 differently regulated lipids, with $p<0.05$ and a fold change between -1.5 and +1.5 (volcano plot in Panel B). Following the decrease of ETI levels in the brain, these alterations virtually disappear, as shown by the Scores Plot a P28 (Panel C) with very few (negligible) alterations of the global lipidome (volcano plot in Panel D).

general upregulation of free fatty acids (FFA) and a corresponding downregulation of acylcarnitines, which transport FFA into mitochondria for subsequent fatty acid oxidation [34]. Based on this finding, we speculate that early exposure to ETI might also have an impact on cell energy metabolism. Moreover, alterations in the brain levels of phosphatidylcholines (PC) and phosphatidylethanolamines (PE) were observed in the pups born from ETI dams compared to control, with a marked upregulation of polyunsaturated species. Interestingly, a similar alteration in brain acylcarnitines has been observed in prenatal exposure of mice to other chemicals, like phthalates [35] and brominated flame retardants [36]. These molecular changes virtually disappear at P28 (Fig. 3, Panel C), with very few significantly altered lipid species observed (Fig. 3, Panel D). This demonstrates that a) the effect of the exposure to ETI is likely not limited to sphingolipid metabolism and dHCer accumulation and b) the effect reverts as the exposure to the drug ceases. It is important to highlight that dHCer increase again with full exposure to Tezacaftor, as demonstrated by the brain dHCer levels we measured in the *dams* (Figure S3): these females have been fed with ETI food for a total of 6 weeks.

3.5. EVALUATION OF PUPS SIZE AND BEHAVIOUR

Based on our molecular data, showing a significant dHCer increase in the brain at P10, we decided to carry out a set of SHIRPA [37,38]

phenotypical and behavioral tests on the newborn pups, to better assess the locomotor and sensory activity of the animals, as well as their physical appearance. We decided to start with the SHIRPA test at P14 to exclude additional stress as a variable in young pups and in the mother's behavior. A careful assessment of weight and size of the animals revealed that HFD+ETI pups are indeed smaller in size, but not in weight, than the HFD control at P14. This discrepancy is recovered at P21 and HFD+ETI and HFD animals are equal in size at P28. This trend is shown in Fig. 4.

The SHIRPA analysis of pups reveals that motor function is not affected by the exposure to ETI at any point of the development. Conversely, the primary reflex and the sensory function at P14 show significant differences in HFD+ETI pups compared to the controls, as shown in Fig. 5.

The SHIRPA tests we conducted on this population revealed that, similarly to what we observed for the pups' size, animals exposed to ETI during pregnancy show a significantly lower performance than the HFD controls for primary reflex and sensory function at P14. This discrepancy is recovered later during breastfeeding and at the beginning of weaning (P21). The SHIRPA results are identical for both groups at P28. Supplementary File 2 reports the full set of SHIRPA phenotypical and behavioral data for P14, P21 and P28.

4. Conclusions

We here describe the changes in the lipidome observed in P10 pups and associated with exposure to ETI of the dams. As shown, the daily administration to female mice during pregnancy results in clear changes in the levels of dihydroceramides, but also several other lipids, in the brain of the newborn pups. No changes were observed in the sciatic nerve, likely due to the very low distribution of Tezacaftor in the PNS compared to the brain (roughly 1/20). In this project, we also paired our molecular investigations with an evaluation of the pups' sensory and locomotor activities. Our data show that the alterations observed revert during breastfeeding, when the exposure to the drug virtually ceases. At P28, pups' size and weight, as well as dHCer and other brain lipids are unaltered compared to controls. We could also speculate that a delay of several days occurs between the exposure to Tezacaftor and an observable accumulation of dHCer in the brain. This because 1) at P28 brain dHCer in ETI pups have not (yet) increased again but 2) around P23 pups begin eating ETI food themselves and 3) at P28 Tezacaftor brain levels increase again to around 1/4 of the levels observed at P10. Following full weaning, with pups becoming adult mice and eating their own food, dHCer accumulates again in the brain, as demonstrated by our previous work[6] and as shown in Figure S3. In the present paper, we also introduce an extensive discussion and characterization of the dose of ETI administered to the animals under investigation. This is a crucial point to derive any further conclusion on our (and other groups) experiments. We have precisely quantified ETI concentration in the dams and we demonstrated (Table 1) that it is comparable to the concentration measured in pwCF undergoing ETI treatment [27,32] and, more importantly, totally in line with the drug levels observed in pregnant CF women [32]. We also demonstrated that these levels are reached in mice only if an allometric scaling of the dose [26] is applied. Indeed, we compared our data with Vertex mice ToxPK data: their 30 mg/Kg dosing arm produced (at 177 days) roughly three times the Tezacaftor concentration we reached in our 56-day-long study, at a practical dose of 17 mg/Kg (based on the measured Tezacaftor incorporation in the food). These data are well in agreement with each other and demonstrate that this range of dosing (20-30 mg/Kg) is the only option reproducing in mice the plasma levels of ETI observed at 1.66 mg/Kg dose in humans (100 mg Tezacaftor tablet to a 60 Kg adult individual). Indeed, in the worst-case scenario, based on Table 1 data, we can state with confidence that the present study is not overdosed, and it demonstrates significant dHCer accumulation in the brain of pups at doses of Tezacaftor *lower* than those observed in humans, thus enforcing the need for further safety assessments of this drug. We also measured the levels of Tezacaftor metabolite M1, which is known to be a CFTR modulator itself

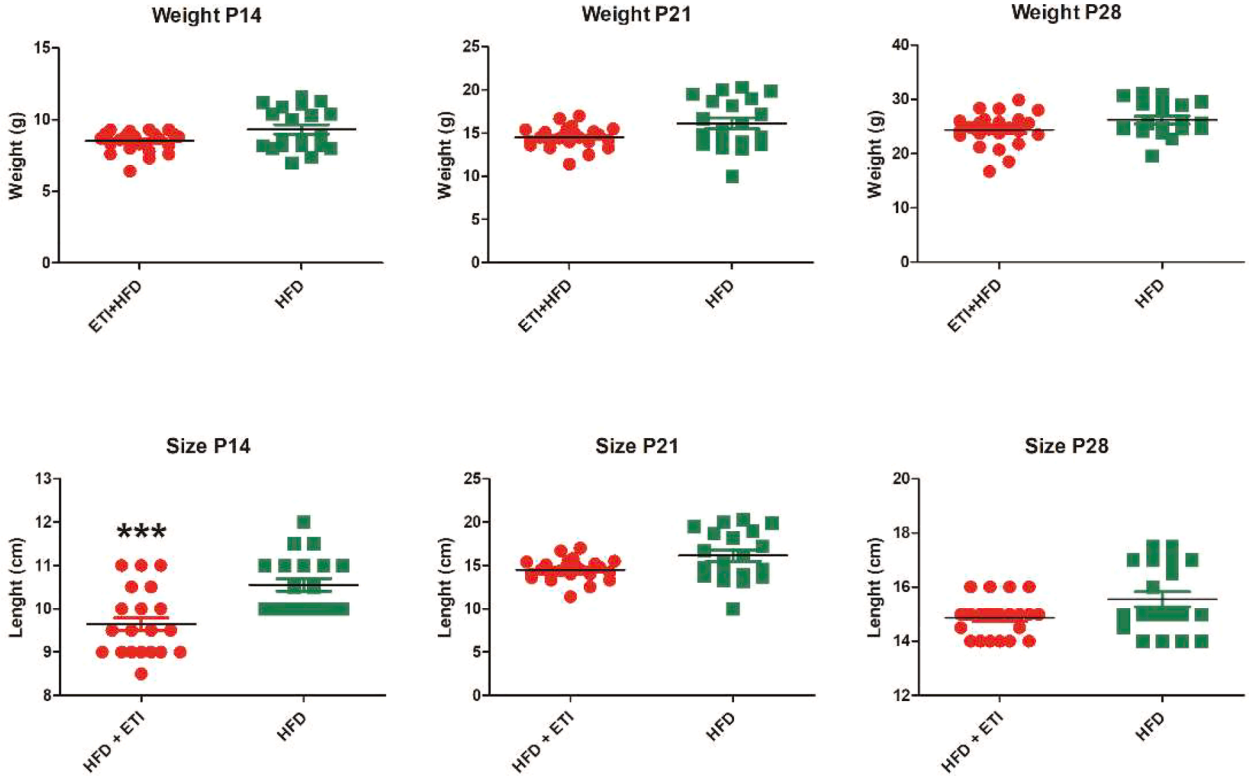

Fig. 4. Comparison of the physical appearance of ETI pups compared to controls in the P14-P28 time window. ETI pups are significantly smaller in size than the controls at P14 (*** $p<0.001$ two-tailed t-test) but recover at P21. At P28 no differences between the two groups are observable.

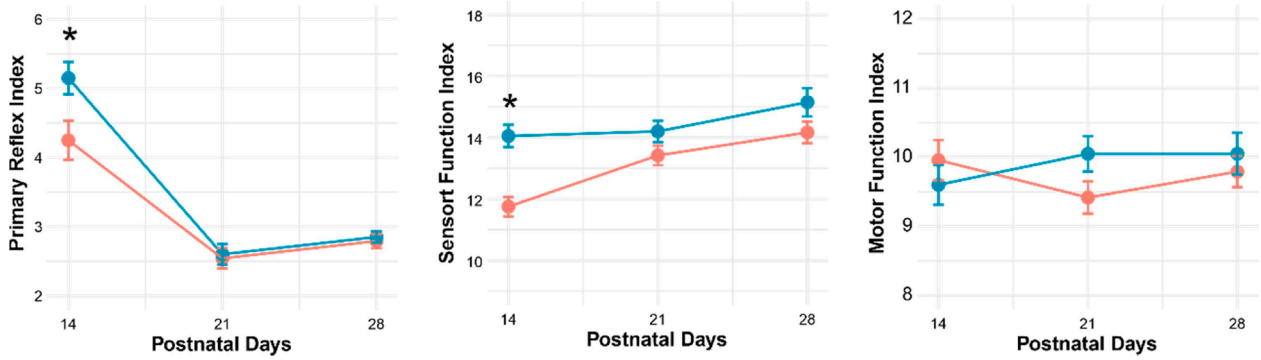

Fig. 5. Results of SHIRPA analysis on HFD+ETI pups compared to controls in the P14-P28 time window for motor function, primary reflex, and sensory function (* $p<0.05$, two-tailed *t*-test).

(Clinical Pharmacology Reviews document, application 210491Orig1s000). The levels of this metabolite are not always reported in papers on ETI quantification in pwCF, despite being often as abundant as the parent drug in both mice and humans (Table 1). Given its chemical structure, we can hypothesize that this compound might also inhibit DEGS, but further experiments are needed on this topic. Nevertheless, irrespective of the inhibitor (Tezacaftor, Tezacaftor-M1 or both), the effects of such inhibition are clearly visible in the brain of ETI pups at P10. From a molecular standpoint, these changes, somehow expected based on our previous work, are clear and quantifiable and might likely go beyond the effect of DEGS inhibitions. It should nevertheless be clearly stated that the molecular data reported here are solely related to the effect of ETI *per se* against the control and should thus be taken with caution, because it is hard to estimate the right metric to evaluate these changes. Fig. 3A, for example, clearly shows that ETI induces observable changes in the brain of pups exposed to ETI compared to controls, but it does not indicate the *magnitude* of such an effect. Much larger changes (in module) might be needed to induce observable phenotypes. Future studies will be aimed to determine if the molecular alterations we observed are related to any possible structural and/or functional alteration, *in primis* in myelin which, as described, might be affected by a prolonged DEGS inhibition. We believe that the data reported here might be useful to stimulate further discussion on the safety of the use of Tezacaftor during pregnancy, perhaps also by investigating potential long-term neurological effects.

Study limitation

The main limitation related to this study is the use of wild-type CD-1 mice, while ETI is given to individuals with CF. However, pregnant women with CF and treated with ETI carry in most cases a non-CF fetus, who is also exposed to the drug. On the other hand, we have already demonstrated that the inhibition of DEGS induced by Tezacaftor is totally CFTR-genotype independent [5,6] and we focused on the safety of the drug *per se*, avoiding potential confounding factors related to the CF pathology. Based on literature [39], CFTR malfunctioning might indeed *reduce* BBB permeability through the action on sphingosine 1-phosphate signaling. We thus do not expect that WT CD-1 mice might have an increased brain penetration of drugs compared to CF animals. On the other hand, similar studies on ETI safety during maternal exposure have been conducted on WT animals, like Sprague-Dawley rats [22, 40,41].

Authorization for animal study

The present study was authorized by the Italian Ministry of Health, N: 1067/2024-PR

CRediT authorship contribution statement

Angelica Squarzoni: Investigation. **Gaia Boschetti:** Investigation. **Sine Mandrup Bertozzi:** Investigation. **Maria Summa:** Investigation. **Angelo Serani:** Investigation. **Elisa Milandri:** Investigation. **Roberto Mandrioli:** Investigation. **Michele Protti:** Investigation. **Laura Mercolini:** Investigation. **Caterina Montani:** Investigation. **Giovanna Capodivento:** Investigation. **Giuliana Cangemi:** Writing – review & editing, Formal analysis. **Nicoletta Pedemonte:** Writing – review & editing, Formal analysis. **Tiziano Bandiera:** Writing – review & editing, Formal analysis. **Fabio Benfenati:** Writing – review & editing, Formal analysis. **Lucilla Nobbio:** Writing – review & editing, Formal analysis. **Rosalia Bertorelli:** Writing – review & editing, Formal analysis. **Andrea Armirotti:** Conceptualization, Supervision, Formal analysis, Writing – original draft.

Declaration of competing interest

The Authors declare that no conflict of interest exists. Andrea Armirotti, Ph.D. (on behalf of all authors)

Acknowledgements

This study received funding from the Cystic Fibrosis Foundation, with grant ARMIRO24G0 to AA and from Fondazione Ricerca Fibrosi Cistica ETS, with grant FFC#2-2024 to LN. NP acknowledges Fondazione per la Ricerca sulla Fibrosi Cistica grant FFC #10/2021 (with the contribution of “Delegazione FFC Ricerca di Genova”, “Delegazione FCC Ricerca Altomilanese”, “Gruppo di sostegno FFC Ricerca di Campiglione Fenile”, and “Delegazione FFC Ricerca di Napoli”). Work in NP and GC Labs is also supported by the Italian Ministry of Health through grant PNRR-MR1-2023-12378412 and through Cinque per mille (5Xmille 2019 grant 5M-2019-23680413) and Ricerca Corrente. EM acknowledges Fondazione Cassa di Risparmio di Imola for supporting her Ph.D. project. We wish to thank Dr. Angelo Reggiani for helpful discussion on allometric dose scaling.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2025.11.004.

References

[1] Aspinall SA, et al. Evaluating the Effect of Kaftrio on Perspectives of Health and Wellbeing in Individuals with Cystic Fibrosis. Int J Env Res Public Health 2022;(10):19.
[2] Graziano S, et al. Longitudinal Effects of Elexacaftor/Tezacaftor/Ivacaftor: Multidimensional Assessment of Neuropsychological Side Effects and Physical and Mental Health Outcomes in Adolescents and Adults. Chest 2024;165(4):800–9.

[3] Papadakis L, et al. Heterogeneity in Reported Side Effects Following Initiation of Elexacaftor-Tezacaftor-Ivacaftor: Experiences at a Quaternary CF Care Center. Pediatr Pulmonol 2025;60(1):e27382.
[4] Spoletini G, et al. Dose adjustments of Elexacaftor/Tezacaftor/Ivacaftor in response to mental health side effects in adults with cystic fibrosis. J Cyst Fibros 2022;21(6):1061–5.
[5] Liessi N, et al. The combination elexacaftor/tezacaftor/ivacaftor (ETI) modulates the de novo synthethic pathway of ceramides in a genotype-independent manner. J Cyst Fibros 2023.
[6] Ciobanu DZ, et al. Tezacaftor is a direct inhibitor of sphingolipid delta-4 desaturase enzyme (DEGS). J Cyst Fibros 2024;23(6):1167–72.
[7] Fabrias G, et al. Dihydroceramide desaturase and dihydrosphingolipids: debutant players in the sphingolipid arena. Prog Lipid Res 2012;51(2):82–94.
[8] Planas-Serra L, et al. Sphingolipid desaturase DEGS1 is essential for mitochondria-associated membrane integrity. J Clin Invest 2023;(10):133.
[9] FDA Kaftrio information file. https://www.ema.europa.eu/en/medicines/human/EPAR/kaftrio#authorisation-details-section; 2021.
[10] EMA KAFTRIO - EPAR Medicine Overview. 2022.
[11] Ripani P, et al. Maternal, newborn and breast milk concentrations of elexacaftor/tezacaftor/ivacaftor in a F508del heterozygous woman with cystic fibrosis following successful pregnancy. Front Med 2023:10.
[12] Blackburn NB, et al. Rare DEGS1 variant significantly alters de novo ceramide synthesis pathway. J Lipid Res 2019;60(9):1630–9.
[13] Dolgin V, et al. DEGS1 variant causes neurological disorder. Eur J Hum Genet 2019;27(11):1668–76.
[14] Karsai G, et al. DEGS1-associated aberrant sphingolipid metabolism impairs nervous system function in humans. J Clin Invest 2019;129(3):1229–39.
[15] Pant DC, et al. Loss of the sphingolipid desaturase DEGS1 causes hypomyelinating leukodystrophy. J Clin Invest 2019;129(3):1240–56.
[16] Capodivento G, et al. Sphingomyelin as a myelin biomarker in CSF of acquired demyelinating neuropathies. Sci Rep 2017;7(1):7831.
[17] Davis DL, et al. Dynamics of sphingolipids and the serine palmitoyltransferase complex in rat oligodendrocytes during myelination. J Lipid Res 2020;61(4):505–22.
[18] Doretto S, et al. Oligodendrocytes as Regulators of Neuronal Networks during Early Postnatal Development. Plos One 2011;6(5).
[19] Kinney HC, et al. Myelination in the developing human brain: Biochemical correlates. Neurochem Res 1994;19(8):983–96.
[20] Tzou FY, et al. The pathophysiological role of dihydroceramide desaturase in the nervous system. Prog Lipid Res 2023;91:101236.
[21] Kaylor JJ, et al. Identification of DES1 as a vitamin A isomerase in Muller glial cells of the retina. Nat Chem Biol 2013;9(1):30–6.
[22] Li D, et al. Fetal drug exposure after maternally administered CFTR modulators Elexacaftor/Tezacaftor/Ivacaftor in a rat model. Biomed Pharmacother 2024;171.
[23] Naskar S, et al. The development of synaptic transmission is time-locked to early social behaviors in rats. Nat Commun 2019;10(1).
[24] Chong J, et al. MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. Nucleic Acids Res 2018;46(W1):W486–94.
[25] Li D, et al. Extent of foetal exposure to maternal elexacaftor/tezacaftor/ivacaftor during pregnancy. Br J Pharmacol 2024;181(15):2413–28.
[26] Nair A, Jacob S. A simple practice guide for dose conversion between animals and human. J Basic Clin Pharm 2016;7(2).
[27] Pigliasco F, et al. Simultaneous Quantification of Ivacaftor, Tezacaftor, and Elexacaftor in Cystic Fibrosis Patients' Plasma by a Novel LC-MS/MS Method. Biomedicines 2023;11(2).
[28] Millán-Santiago J, et al. Volumetric absorptive microsampling for the therapeutic drug monitoring of psychiatric patients treated with cariprazine. J Pharm Biomed Anal 2023:236.
[29] Protti M, Mandrioli R, Mercolini L. Microsampling for therapeutic drug monitoring in psychiatric practice. Int Clin Psychopharmacol 2024;39(1):42–6.
[30] Pigliasco F, et al. A Novel LC-MS/MS Method for the Measurement of Elexacaftor, Tezacaftor and Ivacaftor in Plasma, Dried Plasma Spot (DPS) and Whole Blood in Volumetric Absorptive Microsampling (VAMS) Devices. Pharmaceutics 2025;17(2):200.
[31] Vonk SEM, et al. Dried Blood Spot Method Development and Clinical Validation for the Analysis of Elexacaftor, Elexacaftor-M23, Tezacaftor, Tezacaftor-M1, Ivacaftor, Ivacaftor Carboxylate, and Hydroxymethyl Ivacaftor Using LC-MS/MS. Ther Drug Monit 2024;46(6):804–12.
[32] Christina S, et al. Therapeutic drug monitoring of elexacaftor, tezacaftor, and ivacaftor before, during, and after pregnancy in women with cystic fibrosis: An observational study. Respir Med 2025;236:107868.
[33] Liessi N, et al. Distinctive lipid signatures of bronchial epithelial cells associated with cystic fibrosis drugs, including Trikafta. JCI Insight 2020;5(16).
[34] Longo N, Frigeni M, Pasquali M. Carnitine transport and fatty acid oxidation. Biochim Biophys Acta (BBA) - Mol Cell Res 2016;1863(10):2422–35.
[35] Vacy K, et al. The associations between prenatal plastic phthalate exposure and lipid acylcarnitine levels in humans and mice. Reprod Toxicol 2025:132.
[36] Kramer NE, et al. The brominated flame retardant hexabromocyclododecane causes systemic changes in polyunsaturated fatty acid incorporation in mouse lipids. Toxicol Sci 2025;203(1):118–29.
[37] Lalonde R, Filali M, Strazielle C. SHIRPA as a Neurological Screening Battery in Mice. Curr Protoc 2021;1(5):e135.
[38] Rogers DC, et al. SHIRPA, a protocol for behavioral assessment: validation for longitudinal study of neurological dysfunction in mice. Neurosci Lett 2001;306(1-2):89–92.
[39] Lidington D, et al. CFTR Therapeutics Normalize Cerebral Perfusion Deficits in Mouse Models of Heart Failure and Subarachnoid Hemorrhage. JACC Basic Transl Sci 2019;4(8):940–58.
[40] Li D, et al. Extent of foetal exposure to maternal elexacaftor/tezacaftor/ivacaftor during pregnancy. Br J Pharmacol 2024;181(15):2413–28.
[41] Zhu Y, et al. Lens-related ocular changes in fetal rats following in-utero exposure to elexacaftor-tezacaftor-ivacaftor. J Cyst Fibros 2025;24(1):21–5.