Folate deficiency increases the incidence of dolutegravir-associated foetal defects in a mouse pregnancy model

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eBioMedicine
2023;95: 104762
Published Online xxx
https://doi.org/10.1016/j.ebiom.2023.104762

Summary

Background Dolutegravir (DTG) is a recommended first-line regimen for all people with Human Immunodeficiency Virus (HIV) infection. Initial findings from Botswana, a country with no folate fortification program, showed an elevated prevalence of neural tube defects (NTDs) with peri-conceptional exposure to DTG. Here we explore whether a low folate diet influences the risk of DTG-associated foetal anomalies in a mouse model.

Methods C57BL/6 mice fed a folate-deficient diet for 2 weeks, were mated and then randomly allocated to control (water), or 1xDTG (2.5 mg/kg), or 5xDTG (12.5 mg/kg) both administered orally with 50 mg/kg tenofovir disoproxil fumarate 33.3 mg/kg emtricitabine. Treatment was administered once daily from gestational day (GD) 0.5 to sacrifice (GD15.5). Foetuses were assessed for gross anomalies. Maternal and foetal folate levels were quantified.

Findings 313 litters (103 control, 106 1xDTG, 104 5xDTG) were assessed. Viability, placental weight, and foetal weight did not differ between groups. NTDs were only observed in the DTG groups (litter rate: 0% control; 1.0% 1xDTG; 1.3% 5xDTG). Tail, abdominal wall, limb, craniofacial, and bleeding defects all occurred at higher rates in the DTG groups versus control. Compared with our previous findings on DTG usage in folate-replete mouse pregnancies, folate deficiency was associated with higher rates of several defects, including NTDs, but in the DTG groups only. We observed a severe left-right asymmetry phenotype that was more frequent in DTG groups than controls.

Interpretation Maternal folate deficiency may increase the risk for DTG-associated foetal defects. Periconceptional folic acid supplementation could be considered for women with HIV taking DTG during pregnancy, particularly in countries lacking folate fortification programs.

Funding This project has been funded by Federal funds from the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Department of Health and Human Services, under Contract No. HHSN275201800001I and award #R01HD104553. LS is supported by a Tier 1 Canada Research Chair in Maternal-Child Health and HIV. HM is supported by a Junior Investigator award from the Ontario HIV Treatment Network.

Keywords: INSTI; Foetal anomalies; Neural tube defects; Bleeding; Oedema; Axial asymmetry

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Research in context

Evidence before this study

An elevated rate of neural tube defects (NTDs) with dolutegravir (DTG) use from conception was first reported from the Tsepamo study in Botswana-a country with no folic acid fortification program in place. While this NTD signal has now waned to background levels, similar to those seen in non-DTG based regimens, it is still important to understand whether DTG use is associated with a risk for foetal defects. In this study, we asked whether an interaction may exist between DTG and maternal folate deficiency in enhancing birth defects.

We searched PubMed for relevant prior work based on "dolutegravir AND (neural tube defects OR foetal anomalies) AND (folate OR folic acid)". Serum folate concentrations in pregnant participants in the ADVANCE trial showed slight increases at 12 weeks post DTG initiation. In vitro studies have suggested that DTG is a partial antagonist of folate receptor 1, and DTG exposure was associated with a modest reduction in expression of the folate transporters and decreased uptake of their substrates, although the clinical relevance of this has been questioned. A study in zebrafish reported developmental toxicity with early embryonic exposure to DTG that was rescued with folate supplementation.

Added value of this study

We performed a large prospective mouse DTG fetotoxicity study in mice fed a folate-deficient diet for a minimum of 2 weeks prior to mating. Mice were then randomly allocated to either a control group which received water, a 1xDTG group (therapeutic) which received a dose of DTG yielding plasma levels equivalent to those reported in pregnant women, and a 5xDTG group (supratherapeutic) which received 5× the therapeutic dose. Both DTG groups also received the same nucleoside reverse transcriptase inhibitor (NRTI) backbone to mimic the clinical treatment scenario. We evaluated gross foetal anomalies and maternal and foetal folate levels and profiles. We evaluated 313 litters (2301 foetuses) for anomalies, and observed 8 NTDs in the 1xDTG and 8 NTDs in the 5xDTG group (litter proportion (average of the proportion of foetuses affected per litter) of 1.0% (95% CI 0.21%, 1.8%) and 1.31% (95% CI 0.26%, 2.4%) respectively). No NTDs were observed in the control group. Folate-deficiency was associated with a 2-fold increase in the risk for NTD in the DTG arms, compared with our previously published data using the same model and treatment strategies but under folate-replete conditions. Other defects included abdominal wall, craniofacial, limb, and bleeding defects, all of which were significantly more frequent in the DTG groups versus control, and were increased under folate-deficient conditions in the DTG treated mice. We also observed a severe left-right asymmetry phenotype, reminiscent of limb–body wall complex that was significantly more frequent in the DTG groups and only seen under folate-deficient conditions. Foetal folate concentrations did not differ between treatment arms. This is in contrast to our previous findings under folate-replete conditions, in which the 5xDTG group was associated with higher foetal folate levels and lower rates of foetal defects compared to the 1xDTG group, suggesting a compensatory response that is not achieved in folate-deficient conditions. Additionally, in the 1xDTG group we observed a shift in the foetal folate profiles that suggests the potential for presence of a metabolic 'methyl folate trap', where folates are channelled towards the methylation cycle at the expense of pyrimidine and purine biosynthesis.

Implications of all the available evidence

The results of this study show a potential relationship among DTG, folate, and foetal defects in mice, with diminished maternal folate levels increasing the risk for DTG-associated foetal defects. Our findings support the use of folic acid supplementation in the context of DTG treatment in pregnancy.

Introduction

Dolutegravir (DTG), an integrase strand transfer inhibitor, is a preferred first-line antiretroviral (ARV) due to its efficacy, tolerability, and high barrier to resistance.[1] In 2018, evidence of potential fetotoxicity arose in a nationwide birth surveillance programme in Botswana where infants born to women receiving DTG based antiretroviral therapy (ART) from conception had an elevated risk for neural tube defects (NTDs) (0.94%).[2] With ongoing surveillance in this region the apparent rate of NTDs has declined, with a rate of 0.30% for those taking DTG from conception reported in 2019[3] and 0.11% in 2022.[4] This compares with 0.11% in those taking any non-DTG-based regimen from conception and 0.08% for those without HIV.[3] Additional surveillance data from Botswana reported a NTD rate of 0.66% (1 NTD in 152 deliveries).[5] While Botswana has no public folic acid fortification program in place, a similar surveillance study in Brazil, where folate fortification is underway, reported an estimated 0.18% rate of NTDs among women taking DTG from conception, based on 2 NTDs reported after the closing date of the study.[6] Studies from other countries, including the United States, Canada, and Europe, reported no NTDs, although the samples sizes for these studies were small.[7,8]

Susceptibility to NTDs and other congenital anomalies is influenced by genetic, environmental, and maternal factors, including folate status and exposure to specific drugs.[9,10] Folic acid fortification has reduced the population prevalence of NTDs, and maternal supplementation with folic acid reduces the risk of a

NTD-affected pregnancy, but does not prevent all NTDs.[11–13] Whether there is an association or interaction between DTG-related congenital anomalies and folate status is a research area of interest. A study examining folate levels in serum samples collected from the ADVANCE trial reported that folate levels increased in non-pregnant women taking DTG administered with TAF/FTC over 12 weeks, while folate levels remained stable in women taking DTG/TDF/FTC, and decreased in women taking EFV/TDF/FTC.[14] In the 26 women who became pregnant during the study, serum folate levels increased slightly in those taking DTG/TAF/FTC or DTG/TDF/FTC.[14] *In vitro* studies identified a putative interaction between DTG and folate receptor 1 (FOLR1),[15] with DTG reported to be a partial antagonist of FOLR1.[16] In placental cell lines, DTG exposure was associated with a modest reduction in expression of the folate transporters RFC and PCFT, as well as decreased uptake of their substrates.[17] A modest reduction in expression of Folr1 was reported in gestational day 10.5 placentas from mice treated with DTG, and in first trimester human placenta explants treated *ex vivo* with DTG.[17] Collectively these data may imply that tissue distribution of folate or folate transfer to the foetus could be affected. A study in zebrafish reported developmental toxicity with early embryonic exposure to DTG that was rescued with folate supplementation.[16]

We have previously reported that mice fed a folate-replete diet and treated with a DTG-based regimen at therapeutic dose from conception had higher rates of foetal defects, including a small but significant increase in NTDs, compared to controls.[18] Unexpectedly, mice treated with a supratherapeutic dose of DTG from conception had similar rates of foetal defects as untreated controls. Additionally, foetuses from the dams in the supratherapeutic DTG group had significantly higher total folate levels, possibly indicating induction of a protective mechanism at the higher DTG dose and suggesting that higher foetal folate levels may be protective in the context of DTG treatment. While we found that supratherapeutic DTG treatment under folate-sufficient conditions led to elevated foetal folate content, it is unclear how to interpret reported findings of DTG-associated reduction in folate transporter gene expression/activity and partial antagonism of FOLR.[15–17] Hence it is important to consider the effect of DTG exposure in folate-replete (previous study) and in folate-deficient conditions. In the current study we have addressed this question in a large prospective fetotoxicity study evaluating gross foetal anomalies in mice treated with either therapeutic or supratherapeutic doses of DTG from conception under folate-deficient conditions. Comparison with our previous data under folate-sufficient conditions are provided.[18] We performed this study to examine whether folate deficiency exacerbates DTG-associated foetal anomalies in this model, and whether the protective effects of the supratherapeutic dose of DTG we previously observed are maintained in the context of folate deficiency.

Methods

Mice

C57BL/6J mice bred in-house (original breeders from Jackson Laboratory RRID:IMSR JAX:000664), were maintained under a 12-h light/dark cycle, with *ad libitum* access to food and water. Female mice (6–7 weeks old) were placed on a folate-deficient laboratory diet (Envigo TD. 160606) for a minimum of 2 weeks prior to mating, and continued on this diet for the duration of the experiment. The diet is a synthetic folic acid deficient diet with alcohol extracted casein used to limit background folic acid, and contains 0.1–0.2 mg/kg folic acid. The diet does not include any antibiotics. Mice were trained on gavage with water for a week prior to mating to reduce the potential stress of handling during pregnancy. Following 2 weeks on a folate-deficient diet, mice were mated and presence of a vaginal plug was denoted as gestational day (GD) 0.5. As females became plugged they were consecutively assigned to a cycle of control, 1xDTG, and 5xDTG groups, and housed in cages with 5 dams/cage. This allocation allowed for equal number and temporal distribution across groups. Mice were on the folate deficient diet for an average of 4 weeks at the time of neural tube closure, which we have previously shown is sufficient to cause a decline in maternal folate levels.[19]

Treatment

DTG and the nucleoside reverse transcriptase inhibitors (NRTIs) tenofovir disoproxil fumarate/emtricitabine (TDF/FTC) were purchased as prescription drugs. Drug suspensions were prepared fresh each day. Pills were crushed, suspended in distilled water and sonicated for 10 min, and administered once daily by oral gavage (100 µL/mouse). Details on drug dosing determination have been previously reported.[18,20] The 1xDTG group received 2.5 mg/kg DTG + 50 mg/kg TDF + 33.3 mg/kg FTC, yielding DTG peak plasma concentration of ~3000 ng/mL, Ctrough ~150 ng/mL. The 5xDTG group received 12.5 mg/kg DTG + 50 mg/kg TDF + 33.3 mg/kg FTC, yielding DTG peak plasma concentration of ~12,000 ng/mL, Ctrough ~250 ng/mL. Control mice received 100 µL/mouse of distilled water. On the day of plug detection (GD0.5), plugged mice were randomly allocated to control (water; n = 103 litters, 756 foetuses), 1xDTG (n = 106 litters, 777 foetuses), or 5xDTG (n = 104 litters, 768 foetuses) and were treated once daily until sacrifice.

Sample size calculation

Details of our sample size calculation have been reported.[18] Sample size was decided based on a retrospective study that identified two NTDs out of 158 foetuses (1.3%)

exposed *in utero* to DTG-based ART, versus no NTDs in 187 control foetuses. Based on these data we calculated a sample size of 750 foetuses per arm would give us a power of 0.8 to detect a significant difference between groups. We estimated we would need approximately 100 dams per group to achieve that number of foetuses.

Foetal collection

Dams were euthanized on GD15.5 by CO_2 inhalation. A small number of dams were euthanized on GD14.5 (4/106 in the 1xDTG and 11/104 in the 5xDTG) or 16.5 (4/106 in the 1xDTG and 2/104 in the 5xDTG). A few dams were fed on folate-deficient diet for fewer than 12 days (4/106 in the 1xDTG, 1/104 in the 5xDTG, and 3/103 in the control). The data were reviewed for any associations between these minor protocol deviations and outcome before proceeding with the main analyses of the study. Dissection methodology of the dams, collection of the foetuses and placentae has been previously described.[18] Foetal and placental weights were recorded for each litter using a digital scale. Foetuses were examined under a stereo microscope, digital images were taken, and foetuses were fixed in 10% formalin.

Assessment of crown-rump length, facial dysmorphology and foetal anomalies

For quantitative phenotypic assessment, crown-rump length (CRL) for each foetus, head width (HW), interocular distance (IOD), snout width (SW), upper lip length (ULL) were measured and recorded from digital images using ImageJ (Version 1.47t). Images were scored for foetal macroscopic malformations by two independent investigators blinded to the treatment allocation (LS and AC). Disparities between reviewers were resolved by discussion with a third reviewer (NG) prior to unblinding.

Micro-CT imaging

Embryos were subjected to hydrogel stabilization before imaging.[21] Briefly, each embryo was placed in 20 mL hydrogel solution: ice-cold 4% paraformaldehyde (wt), 0.05% (wt/vol) bis-acrylamide (Bio-Rad, Mississauga, ON, Canada), 0.25% VA044 Initiator (Wako Chemicals USA, Inc., Richmond, VA, USA), 0.05% (wt/vol) saponin (Sigma–Aldrich, St Louis, MO, USA) and PBS at 4 °C for 3 days, then placed in a desiccation chamber where air was replaced with nitrogen gas, followed by placement in a 37 °C water bath for 3 h. The sample was then separated from the encasing gel and placed into 50 mL of 0.1 N iodine solution (Sigma–Aldrich) for 24 h, washed in PBS for 1 h, and embedded in 1% agarose prior to imaging.

Three-dimensional data sets were acquired for mouse embryos using a Bruker Skyscan 1272 micro-CT scanner (Bruker Skyscan, Antwerp, Belgium), with the X-ray source at 100 kV and 100 μA. The specimen was rotated 360° in 0.3° increments, generating 1200 views that were reconstructed into data blocks with a 11-μm voxel size. The 3D video models for the embryos were created using the Amira Software.

Folate measurements

Folate analysis of whole embryos and maternal liver collected on GD11.5 was performed by UPLC-MS/MS as described previously.[18,22,23] Folates were measured by multiple reaction monitoring (MRM) which was optimized using cone voltage and collision energy for precursor and product ions.[24] Analysis of the peak areas were carried out using MassLynx software (Waters Corporation).

Statistical analysis

Viability, foetal weights, placental weights, measurements for crown-rump length and facial dysmorphology are presented as litter averages for litters collected at GD15.5 fed on folate-deficient diet for a minimum of 14 days prior to mating. For all dams, defects are reported at the foetal level as frequencies (% of foetuses showing the defect), at the litter level as number of litters that include at least one foetus showing the defect (% of litters affected), and as the litter proportion (by calculating the mean of the proportion of foetuses in each litter with the defect) with 95% confidence intervals (CI) using the normal distribution. Kruskal–Wallis test with Dunn's post-test was used to compare litter proportions of each defect between treatment arms using R (version 4.1.2). Odds ratios (OR) with 95% CI were calculated using mixed effects logistic regression with treatment as a fixed effect and litter as a random effect using STATA (version 13.0). OR were only calculated when at least 5 observations were present in each group. Generalized linear regression models were used to calculate the mean difference with 95% CI for each of the facial dysmorphology and CRL measures versus control using STATA. Folate profile data were analysed using Kruskal–Wallis test with Dunn's post-test in GraphPad Prism (v 8.0).

Ethics

All animal experiments were approved by the University Health Network Animal Care Committee (protocol #2575.26) and performed according to the policies and guidelines of the Canadian Council on Animal Care.

Role of funding source

The study design was approved by the Funder. The funders played no role in data collection, analysis, or interpretation of the data, or drafting of the manuscript. The corresponding and first authors had full access to all the data in the study. The corresponding author had final responsibility for the decision to submit for publication.

Results

Maternal and foetal outcomes

A study schematic is shown in Fig. 1a. Mice fed a folate-deficient diet for a minimum of 2 weeks were mated and on day of plug detection (GD0.5) randomly allocated to either the control group (n = 103) that received water, the 1xDTG group (n = 106) that received 2.5 mg/kg DTG in combination with 50/33 mg/kg TDF/FTC (yielding peak DTG plasma levels of ~3,000 ng/mL), or the 5xDTG group (n = 104) that received 12.5 mg/kg DTG in combination with 50/33 mg/kg TDF/FTC (yielding peak DTG plasma levels of ~12,000 ng/mL). Mice continued on a folate-deficient diet for the duration of the experiment and treatments were administered by oral gavage once daily until sacrifice on GD15.5. The median time on the folate-deficient diet at day of plug was 21 days for all three treatment arms (interquartile range for control: 15–30; for 1xDTG: 17–27; for 5xDTG: 16–24). Comparisons are included from our previously published data using the same experimental set-up under folate-sufficient conditions.[18]

Foetal viability (control: 91.4%; 1xDTG: 89.5%; 5xDTG: 90.9%), foetal resorption (spontaneous abortion equivalent) (control: 8.2%; 1xDTG: 10%; 5xDTG: 8.8%), and foetal non-viability (stillbirth equivalent) (control: 0.43%; 1xDTG: 0.47%; 5xDTG: 0.26%) rates (Fig. 1b) did not differ significantly between groups, or by folate status. Litter size also did not differ significantly between groups (median [IQR]: 8 [7–9] for all groups), and was the same as that seen in the folate-sufficient mice.

Maternal weight gain (Fig. 1c) did not differ significantly between groups in the folate-deficient mice, or by folate-status for any of the treatment arms.

Litter average foetal weight (Fig. 1d) and placental weight (Fig. 1e) did not differ significantly between treatment arms in the folate-deficient mice. As expected, litter average foetal and placenta weights were lower in the control mice on a folate-deficient diet compared to those on a folate-sufficient diet. Foetal and placenta weights in the 1xDTG treatment arm did not differ significantly by folate-status. However, the largest reduction in foetal (–7%) and placenta (–13%) weights was observed in the 5xDTG folate-deficient compared to the 5xDTG folate-sufficient group.

Developmental retardation, where a foetus is scored as being at an earlier stage in development than appropriate, was significantly more frequent in the DTG groups versus control (Table 1).

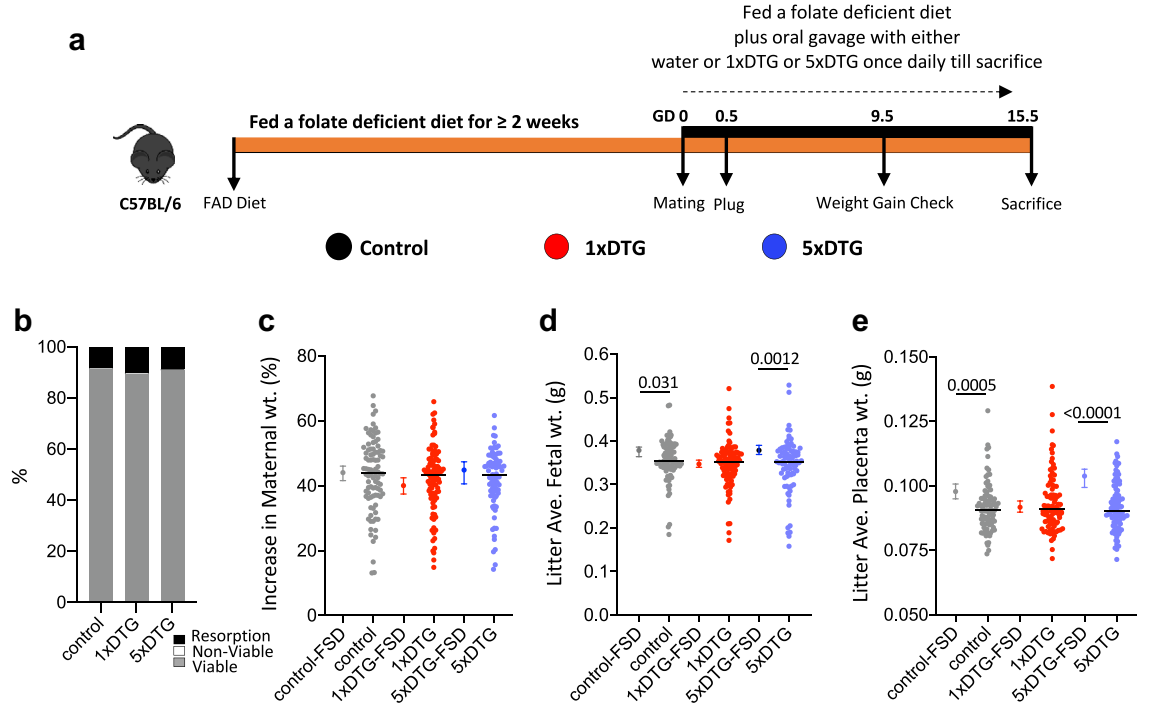

Fig. 1: **Comparison of pregnancy outcomes between treatment arms**. (a) Schematic representation of the experimental design using a folate-deficient diet. Foetal viability and resorptions are shown in (b), percent increase in maternal weight is shown in (c), litter average foetal weight in (d), and litter average placenta weight in (e). Data are shown as dot plots with a line indicating the median for the data relating to the mice fed a folate-deficient diet. Data from the mice fed a folate-sufficient diet (FSD) are shown as a median with 95% confidence interval. Control shown in grey, 1xDTG shown in red, and 5xDTG shown in blue. Statistical comparisons by Kruskal–Wallis with Dunn's post-test. For the folate-deficient groups n = 88 litters for control, 96 litters for 1xDTG, and 82 litters for 5xDTG. Only litters collected at GD15.5 are included in (c), (d), and (e). DTG, Dolutegravir.

	Control N (litter) = 103, N (foetus) = 756			1xDTG N (litter) = 106, N (foetus) = 777			5xDTG N (litter) = 104, N (foetus) = 768		
	Foetuses affected n (%)	Litters affected n (%)	Litter proportion % (95% CI)	Foetuses affected n (%)	Litters affected n (%)	Litter proportion % (95% CI)	Foetuses affected n (%)	Litters affected n (%)	Litter proportion % (95% CI)
Neural tube defects									
Neural tube defects[a]	0 (0%)	0 (0%)	0% (0, 0)	8 (1.0%)	7 (6.6%)	1.0%[b] (0.21, 1.8)[c]	8 (1.0%)	7 (6.7%)	1.3% (0.26, 2.4)[c]
Tail flexion, kinky tail, curly tail	17 (2.2%)	17 (16.5%)	2.1% (1.1, 3.0)	28 (3.6%)	20 (18.9%)	3.8% (1.8, 5.8)	43 (5.6%)	32 (30.8%)	6.5% (3.8, 9.1)[d]
Severe defects									
Severe left-right asymmetry	3 (0.40%)	2 (1.9%)	0.46% (0, 1.2)	17 (2.2%)	10 (9.4%)	2.4% (0.79, 4.0)[c]	6 (0.78%)	4 (3.8%)	1.1% (0, 2.2)
Other severe deformities	0 (0%)	0 (0%)	0% (0, 0)	3 (0.39%)	3 (2.8%)	0.31% (0, 0.67)	3 (0.39%)	3 (2.9%)	0.43% (0, 0.93)
Craniofacial anomalies									
Any craniofacial anomaly	6 (0.79%)	6 (5.8%)	0.69% (0.14, 1.2)	12 (1.5%)	12 (11.3%)	1.6% (0.7, 2.6)	27 (3.5%)	19 (18.3%)	4.4% (2.1, 6.7)[d]
Facial anomaly	0 (0%)	0 (0%)	0% (0, 0)	8 (1.0%)	8 (7.6%)	1.2% (0.34, 2.0)	16 (2.1%)	12 (11.5%)	2.6% (1.0, 4.1)[d]
Oro-facial clefts	0 (0%)	0 (0%)	0% (0, 0)	2 (0.26%)	2 (1.9%)	0.28% (0, 0.7)	3 (0.39%)	3 (2.9%)	0.36% (0, 0.78)
Mandible/maxilla aplasia	6 (0.79%)	6 (5.8%)	0.69% (0.14, 1.2)	2 (0.26%)	2 (1.9%)	0.20% (0, 0.48)	9 (1.2%)	8 (7.7%)	1.6% (0.32, 2.8)
Eye defects									
Any eye defects	47 (6.2%)	39 (37.9%)	6.3% (4.5, 8.0)	44 (5.7%)	28 (26.4%)	6.5% (3.7, 9.3)	31 (4.0%)	25 (24.0%)	3.9% (2.2, 5.5)[c]
Anophthalmia	5 (0.66%)	5 (4.8%)	0.69% (0.08, 1.3)	1 (0.13%)	1 (0.94%)	0.09% (0, 0.28)	3 (0.39%)	3 (2.9%)	0.35% (0, 0.76)
Microphthalmia	30 (4.0%)	26 (25.2%)	4.0% (2.5, 5.4)	32 (4.1%)	20 (18.9%)	5.1% (2.4, 7.8)	19 (2.5%)	17 (16.4%)	2.3% (1.2, 3.4)
Coloboma	14 (1.8%)	13 (12.6%)	1.9% (0.87, 3.0)	11 (1.4%)	10 (9.4%)	1.3% (0.5, 2.1)	9 (1.2%)	8 (7.7%)	1.2% (0.26, 2.2)
Abdominal wall defects									
Gastroschisis, omphalocele	3 (0.40%)	2 (1.9%)	0.46% (0, 1.2)	28 (3.6%)	15 (14.2%)	5.0% (2.0, 7.9)[d]	23 (3.0%)	15 (14.4%)	4.2% (1.4, 7.0)[d]
Limb defects									
Limb defects	4 (0.53%)	3 (2.9%)	0.58% (0, 1.3)	31 (4.0%)	23 (21.7%)	4.3% (2.2, 6.4)[e]	40 (5.2%)	27 (26.0%)	6.1% (3.4, 8.7)[e]
Vascular/bleeding defects									
Cranial bleed	11 (1.5%)	9 (8.7%)	1.4% (0.45, 2.3)	37 (4.8%)	29 (27.4%)	5.3% (2.9, 7.7)[d]	50 (6.5%)	32 (30.8%)	6.2% (4.0, 8.5)[e]
Spinal bleed	24 (3.2%)	19 (18.4%)	3.0% (1.6, 4.4)	83 (10.7%)	50 (47.2%)	11.3% (7.9, 14.6)[e]	94 (12.4%)	58 (55.8)	12.5% (9.7, 15.3)[e]
Petechiae	66 (8.7%)	40 (38.8%)	8.9% (6.1, 11.6)	103 (13.3%)	52 (49.1%)	14.3% (10.2, 18.3)	102 (13.3%)	53 (51.0%)	15.0% (11.1, 19.0)[c]
Haemorrhagic	1 (0.13%)	1 (0.97%)	0.10% (0, 0.29)	8 (1.0%)	8 (7.6%)	1.0% (0.32, 1.8)	11 (1.4%)	7 (6.7%)	1.3% (0.2, 2.4)
Other defects									
Severe oedema	10 (1.3%)	9 (8.7%)	1.2% (0.38, 2.0)	68 (8.8%)	43 (40.6%)	9.0% (5.8, 12.1)[e]	58 (7.6%)	32 (30.8%)	8.1% (4.8,11.4)[e]
Body asymmetry	14 (1.8%)	11 (10.7%)	1.9% (0.63, 3.2)	48 (6.2%)	30 (28.3%)	6.3% (4.0, 8.7)[d]	40 (5.2%)	30 (28.8%)	5.3% (3.4, 7.2)[d]
Short tail	1 (0.13%)	1 (0.97%)	0.14% (0, 0.41)	12 (1.5%)	11 (10.4%)	1.3% (0.51, 2.0)[c]	13 (1.7%)	11 (10.6%)	2.0% (0.76, 3.2)[c]
Skin (peeling/loose)	7 (0.93%)	5 (4.8%)	0.78% (0.01, 1.6)	12 (1.5%)	10 (9.4%)	1.3% (0.5, 2.2)	31 (4.0%)	22 (21.2%)	4.2% (2.4, 6.1)[e]
Growth retarded	14 (1.8%)	10 (9.7%)	3.5% (0.52, 6.4)	60 (7.7%)	30 (28.3%)	9.0% (4.7, 13.4)[d]	56 (7.3%)	24 (23.1%)	9.0% (4.3, 13.6)[c]

[a]The neural tube defects in the 1xDTG group were: 3 exencephalies, 3 encephaloceles, and 2 spina bifidas. The neural tube defects in the 5xDTG were: 4 exencephalies, 2 encephaloceles, and 2 spina bifidas. [b]For percent litter average statistical comparisons between treatment groups by Kruskal–Wallis test with Dunn's multiple comparison post-test. [c]$p < 0.05$. [d]$p < 0.01$. [e]$p < 0.001$ versus control. DTG, Dolutegravir.

Table 1: **Foetal anomalies in mice fed a folate deficient diet and treated with either water (control), 1xDTG, or 5xDTG.**

Crown-rump length and facial dysmorphology

Quantitative assessment of CRL (Fig. 2a) and facial morphology (head width (HW), IOD, snout width (SW), ULL; Fig. 2b) was conducted in a sub-group of foetuses with suitable images for this analysis. CRL was significantly lower in the 1xDTG and 5xDTG compared to control (Fig. 2a). Head width was significantly higher and there was a trend towards higher IOD and snout width in the 1xDTG group compared to control. Head width, IOD, and snout width did not differ significantly between the 5xDTG and control groups (Fig. 2b). No data were available for foetuses under folate-sufficient conditions.

Neural tube defects are more common with DTG treatment

Eight foetuses from 7 litters in the 1xDTG group exhibited NTDs, giving a rate of 1.0% (8/777). These included 3 foetuses with exencephaly (Fig. 3b), 3 with encephalocele (Fig. 3c), and 2 with spina bifida (a normal foetus is shown in Fig. 3a as reference). Eight foetuses from 7 litters in the 5xDTG also exhibited NTDs, with a rate of 1.0% (8/768). These included 4 with exencephaly, 2 with encephalocele, and 2 with spina bifida. No NTDs (0/756) were detected in the control group (Table 1). The litter proportion for NTDs

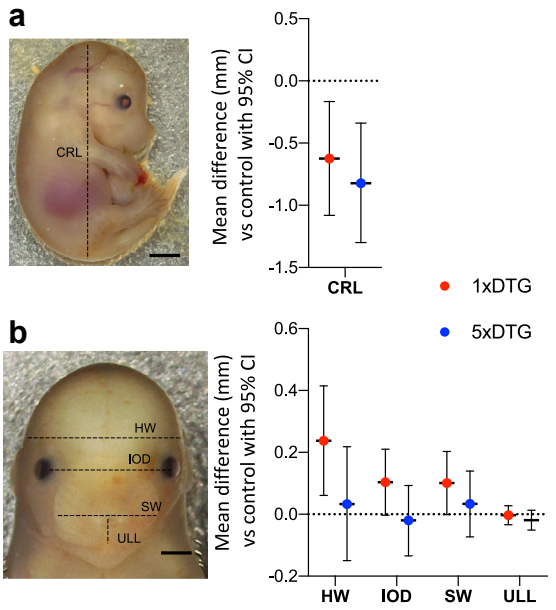

Fig. 2: **Crown-rump length and facial morphometry measure.** Foetuses with suitable images were scored for crown-rump length in (a) and head width (HW), interocular distance (IOD), snout width (SW), and upper lip length in (b). Data are shown as the mean difference from control with 95% confidence interval. 1xDTG is shown in red, 5xDTG in blue. All data are for mice fed a folate-deficient diet. The dotted line represents the control. n = 76 litters for control, 90 for 1xDTG, and 75 for 5xDTG. DTG, Dolutegravir.

(calculated by averaging the percent of foetuses per litter with the defect[25]) was highest in the 5xDTG group at 1.3%, followed by 1.0% in the 1xDTG, and 0% in the control group (Fig. 4a).

Compared with our previous study of mice on a folate-sufficient diet, folate-deficiency was associated with a doubling of the rate of NTDs in the 1xDTG group, although this did not reach statistical significance ($p = 0.15$, by Fisher's exact) (Fig. 4a). The NTD rate in the 5xDTG group was significantly higher in folate-deficient litters (1.3%) compared with folate-sufficient pregnancies (0%). Importantly, we observed indications of a dose–response relationship for NTDs in folate-deficiency (1.0% in the 1xDTG versus 1.3% in the 5xDTG), although this would require more than two doses to establish definitively. This contrasts with the lack of dose response previously observed under folate-sufficient conditions (0.47% in the 1xDTG versus 0% in the 5xDTG).

Under folate-deficient conditions tail defects, specifically flexed tail, kinked tail, or curly tail, were observed at a higher rate in the 1xDTG group (litter proportion 3.8%) versus control (litter proportion 2.1%), and at a significantly higher rate in the 5xDTG (litter proportion 6.5%) group versus control (Table 1). These abnormalities are part of the phenotype of several mouse NTD models and are indicators of delayed spinal neurulation and/or spinal dysraphism.[26] We also calculated the odds ratio (OR) of observing a

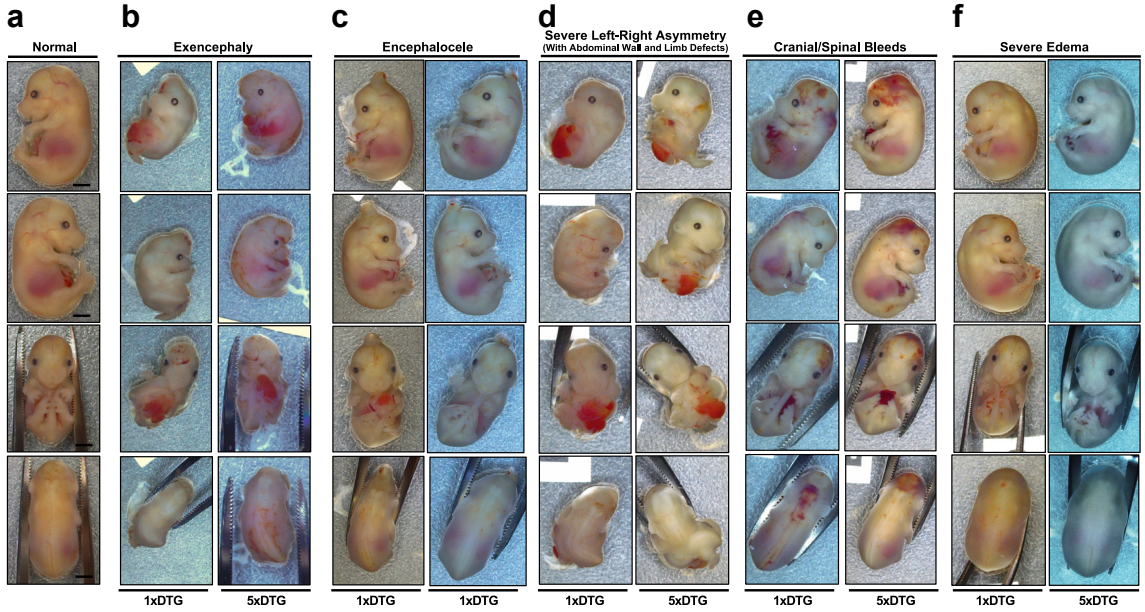

Fig. 3: **Representative images of observed foetal defects.** (a) Normal foetus shown from left, right, front and back views (top to bottom images) (b–f) Representative foetuses with exencephaly (b), encephalocele (c), severe left-right asymmetry (d), cranial/spinal bleeds (e), and severe oedema (f). Each foetus is shown from left, right, front and back views (top to bottom). Foetuses from the 1xDTG group are shown in the left columns for each defect, and the 5xDTG group in the right columns. All mice were fed a folate-deficient diet. Scale bar in (a) represents 1 mm for all images. DTG, Dolutegravir.

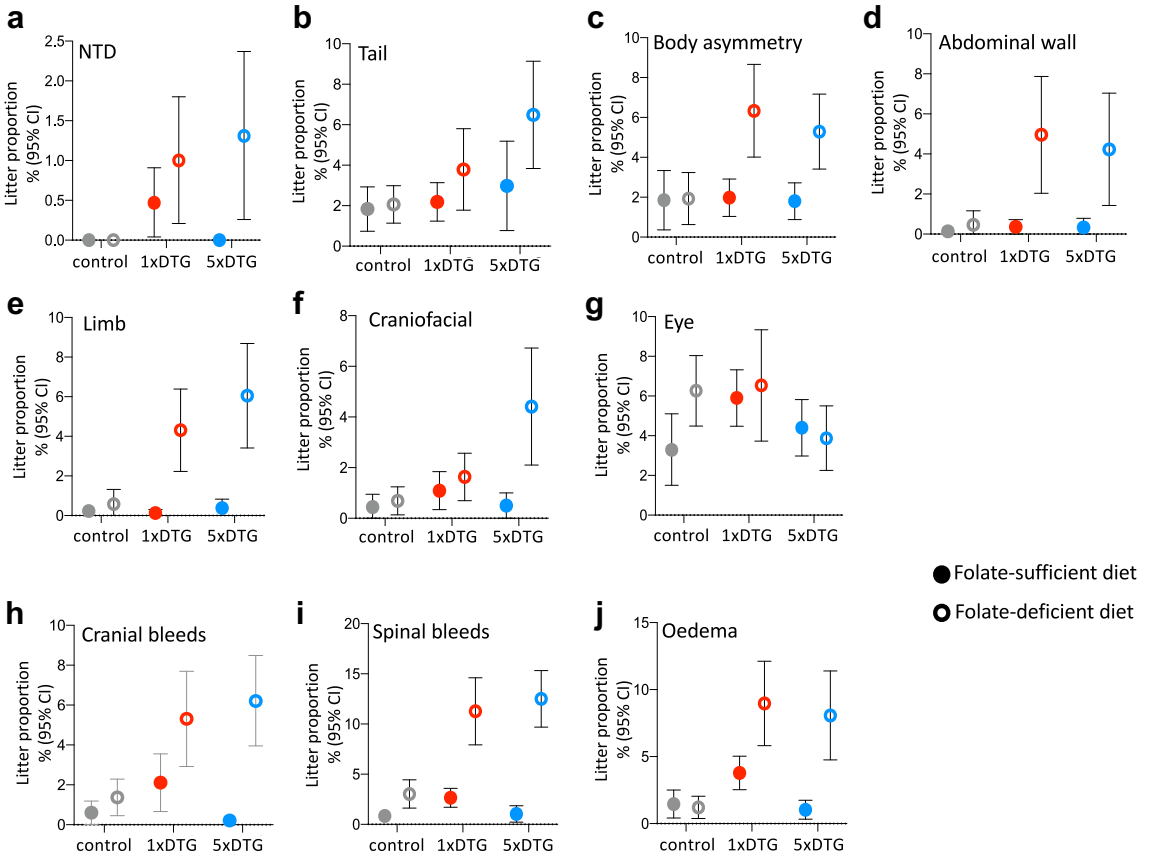

***Fig. 4*: Comparison of various foetal defect rates between control, 1xDTG, and 5xDTG in mice fed either a folate-sufficient or a folate-deficient diet**. (a) Neural tube defects (NTD). (b) Tail defects (curled, kinked, or flexed). (c) Body asymmetry. (d) Abdominal wall defects (gastroschisis/omphalocele). (e) Limb defects. (f) Craniofacial defects. (g) Eye defects. (h) Cranial bleeds. (i) Spinal bleeds (j) Severe oedema. Data are shown as litter proportion (the average of the proportion of foetuses affected per litter) with 95% confidence intervals. The closed dots are data from mice fed a folate-sufficient diet. Open dots are data from mice fed a folate-deficient diet. Control treated mice are shown in grey, 1xDTG treated mice in red, and 5xDTG treated mice in blue. For the mice fed a folate-sufficient diet n = 91 litters for control, 150 for 1xDTG, 111 litters for 5xDTG. For the mice fed a folate-deficient diet n = 103 for control, 106 for 1xDTG, and 104 for 5xDTG. DTG, Dolutegravir.

defect in a DTG group versus control accounting for intra-litter variability using a mixed effects logistic regression that included treatment as a fixed effect and litter as a random effect. This was done only for defects that had at least 5 observations in each group. The odds ratio (OR) of observing a tail defect versus control was 1.6 (95% CI 0.8, 3.3) and 2.8 (95% CI 1.4, 5.6) in the 1xDTG and 5xDTG groups respectively (Supplemental Table S1). Rates of tail defects were higher under folate-deficient than under folate-sufficient conditions in the DTG groups (Fig. 4b).

Severe left-right asymmetry, abdominal wall, and limb defects are more frequent with DTG treatment under folate deficiency

Severe left-right asymmetry defect was observed in all groups but was significantly more frequent in the 1xDTG group versus control. The defect is a complex anomaly affecting the whole body axis (Figs. 3d and 5, and Supplemental Movie Files: 1–5) that resembles human limb-body wall complex[27]–a combination of multiple congenital anomalies that include exencephaly, encephalocele, scoliosis, gastroschisis and limb defects. Seventeen foetuses from 10 different litters of the 1xDTG group exhibited severe left-right asymmetry defect, for an incidence rate of 2.2% (17/777) and a litter proportion of 2.4%. This compared with six foetuses from 4 litters in the 5xDTG group, for an incidence rate of 0.78% (6/768) and a litter proportion of 1.1%. Three foetuses from 2 litters in the control group had this defect, for an incidence of 0.40% (3/756) and a litter proportion of 0.46% (Table 1). This type of severe left-right asymmetry defect was not observed under folate-sufficient conditions.

Less severe axial defects (body asymmetry) were also observed at a significantly higher rate in both DTG

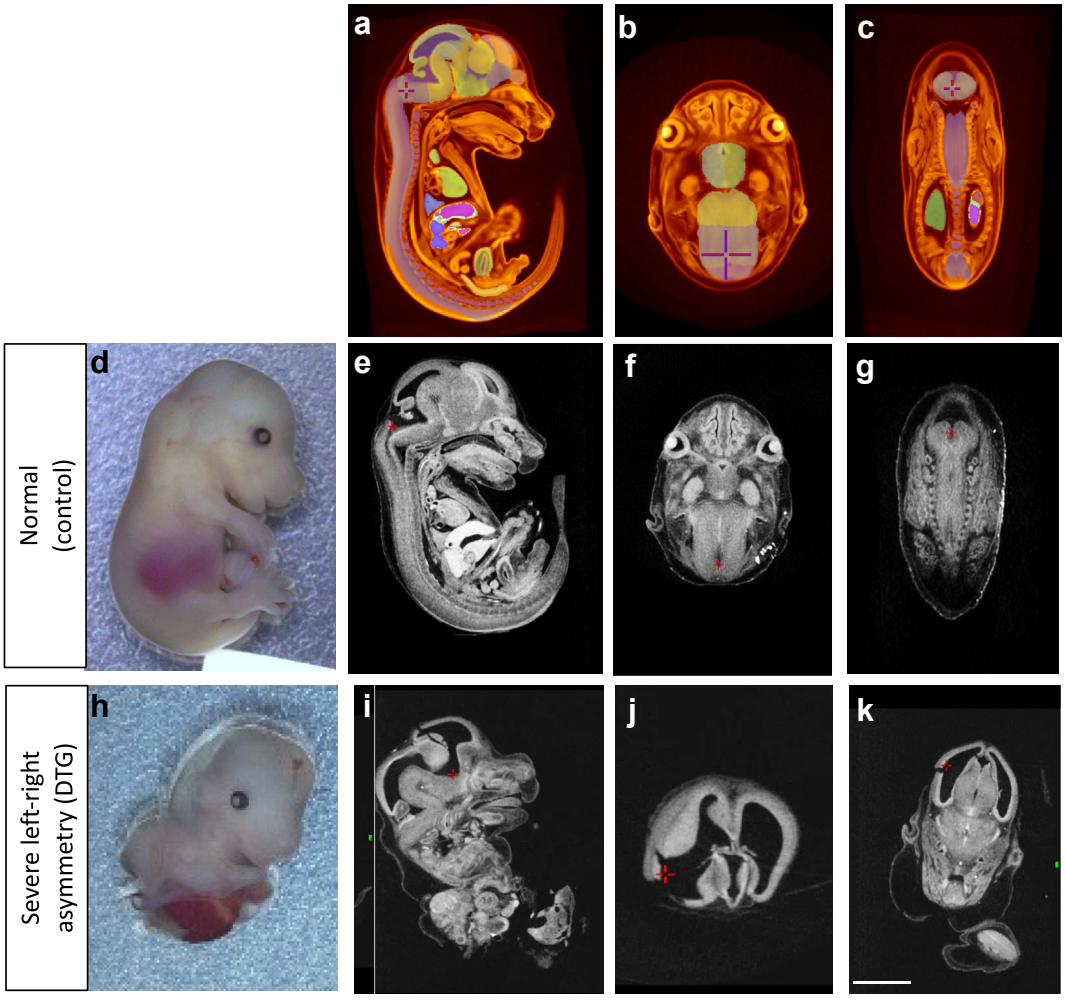

Fig. 5: **Micro-CT image of foetus with severe left-right asymmetry showing brain anomalies**. Three-dimensional visualization of the segmented anatomical structures (a–c) in the E15.5 mouse embryo atlas, in which different anatomical regions are shown by different colours in the embryo. Gross images of the control (d) and DTG treated foetus from a folate-deficient diet (h) are depicted with their corresponding micro-CT images on the right. The sagittal (a, e, i), axial (b, f, j) and coronal (c, g, k), planes are shown respectively. The red cross-hairs represent the corresponding point in all sections for a given embryo. DTG, Dolutegravir.

groups versus control under folate-deficient conditions (Table 1). The OR of observing body asymmetry defects versus control was 3.9 (95% CI 1.8, 8.4) and 3.3 (95% CI 1.5, 7.2) in the 1xDTG and 5xDTG groups respectively (Supplemental Table). Rates of body asymmetry were significantly higher under folate-deficient versus folate-sufficient conditions for the DTG-treated groups, but not for the controls (Fig. 4c).

Abdominal wall defects were significantly more frequent in the 1xDTG and 5xDTG groups (litter proportion 5.0% in the 1xDTG, 4.2% in the 5xDTG group) compared with 0.46% in the control group (Table 1). Folate deficiency was associated with a significant increase in abdominal wall defects compared to the folate-sufficient state in the DTG-treated groups, but not in the control group (Fig. 4d).

Limb defects were significantly more frequent in the DTG-treated groups compared to control (Table 1). As with the abdominal defects, folate deficiency was associated with significantly more limb defects compared to the folate-sufficient state in the DTG groups, but not for the control group (Fig. 4e).

Other anomalies

Craniofacial anomalies (including facial defects, orofacial clefts, and mandibular or maxillary aplasia) showed a dose-dependent increase in the DTG groups versus control reaching significance in the 5xDTG group (litter proportion: 4.4% in 5xDTG versus 1.6% in 1xDTG versus 0.69% in control, Table 1) under folate-deficient conditions. The OR of observing craniofacial anomalies versus control was 2.1 (95% CI 0.7, 6.6) and

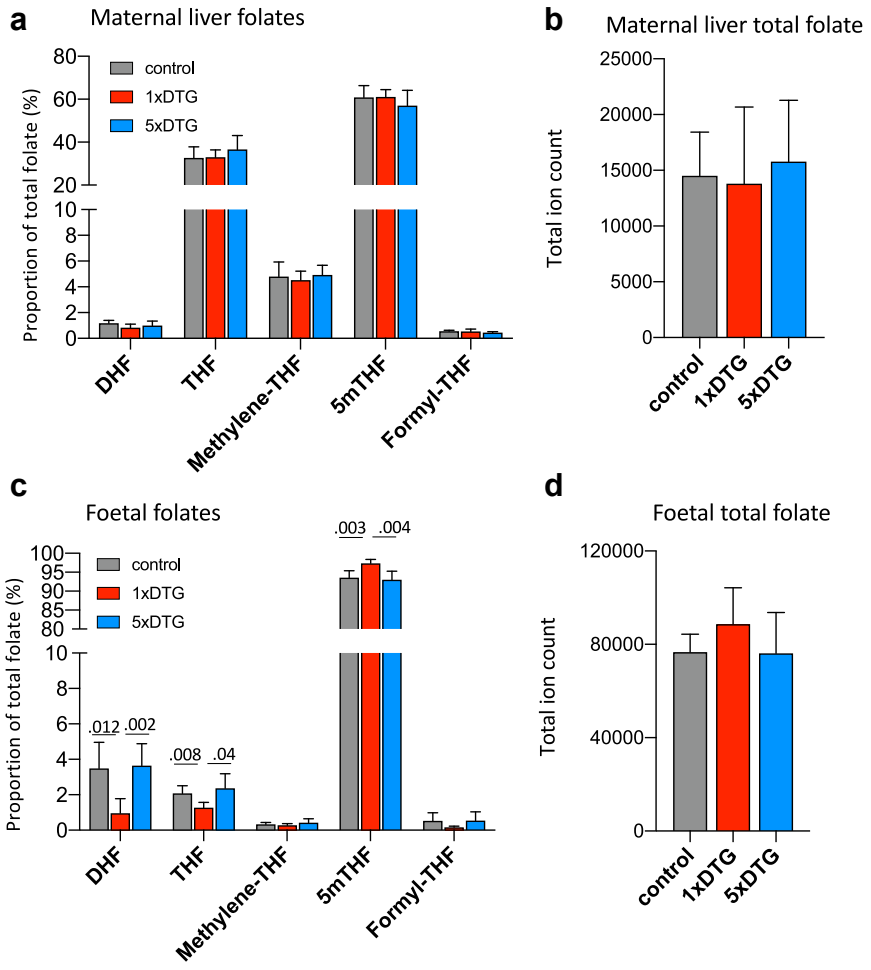

Fig. 6: **Maternal liver and whole foetus folate profiles and total folate levels.** (a) Maternal liver folate profile, and (b) maternal liver total folate levels for mice fed a folate-deficient diet. (c) Whole foetus folate profile, and (d) whole foetus total folates for mice fed a folate-deficient diet. Data are shown as means with 95% confidence intervals. Statistical comparisons by Kruskal–Wallis with Dunn's multiple comparisons post-test. DHF, dihydrofolate; THF, tetrahydrofolate; 5mTHF, methyltetrahydrofolate. For maternal folates n = 5 dams for control, n = 6 for 1xDTG, n = 7 for 5xDTG. Two foetuses per dam were included in the foetal folate analyses, n = 10 for control, n = 12 for 1xDTG, n = 14 for 5xDTG. DTG, Dolutegravir.

5.1 (95% CI 1.8, 14.5) in the 1xDTG and 5xDTG groups respectively (Supplemental Table). Folate deficiency increased the rate of craniofacial anomalies associated with DTG treatment, with defects being significantly more frequent in the folate-deficient versus folate-sufficient 5xDTG group (Fig. 4f).

Eye defects included anophthalmia, microphthalmia, and coloboma. Compared to the control, foetuses in the 5xDTG group were significantly less likely to display eye defects (Table 1). The OR of observing eye defects versus control were 0.8 (95% CI 0.5, 1.5) and 0.6 (95% CI 0.3, 1.1) in the 1xDTG and 5xDTG groups respectively (Supplemental Table). The rate of eye defects did not differ significantly between folate-deficient and sufficient conditions for both DTG groups, but folate-deficiency was associated with more frequent eye defects in the control group (Fig. 4g).

Other anomalies included short tail and skin defects (loose or peeling skin), both of which were more common in the DTG groups versus control (Table 1).

Vascular/bleeding defects and severe oedema

Foetuses in the 1xDTG and 5xDTG were significantly more likely to display cranial or spinal bleeds (Fig. 3e) than the control group. The litter proportion for cranial bleeds was 6.2% in the 5xDTG, 5.3% in the 1xDTG group and 1.4% in the control group. The OR of observing cranial bleed versus control was 3.7 (95% CI 1.7, 8.2) and 5.0 (95% CI 2.3, 10.9) in the 1xDTG and 5xDTG groups respectively (Supplemental Table).

Similarly, the litter proportion for spinal bleeds was 12.5% in the 5xDTG, 11.3% in the 1xDTG group, and 3.0% in the control group (OR versus control 3.9 (95% CI 2.2, 6.9) and 4.8 (95% CI 2.7, 8.4) in the 1xDTG and 5xDTG groups respectively (Supplemental Table)). Both cranial and spinal bleeds were more frequent under folate-deficient versus folate-sufficient conditions, although the difference was more pronounced in the DTG groups (Fig. 4h and i). We previously observed a protective effect against cranial and spinal bleeds in the 5xDTG versus 1xDTG groups under folate-sufficient conditions. This protective effect was not present under folate-deficient conditions (Fig. 4h and i).

Additional bleeding defects included petechia-like bleeds and haemorrhagic foetuses, both of which were more common in the DTG groups versus control, but reached significance only for the petechial bleeds in the 5xDTG vesus control (Table 1, Supplemental Table).

Severe oedema (Fig. 3f, Table 1) was significantly more frequent in the DTG groups versus control (litter proportion 1.2% in the control, 9.0% in the 1xDTG, 8.1% in the 5xDTG). The OR of observing severe oedema versus control was 9.9 (95% CI 4.0, 24) and 7.5 (95% CI 3.0, 7.2) in the 1xDTG and 5xDTG groups respectively (Supplemental Table). Severe oedema rates were significantly higher in the DTG groups under folate-deficient versus folate-sufficient conditions and, as with the bleeding defects, the protective effect observed in the 5xDTG group under folate-sufficiency was not observed under folate-deficient conditions (Fig. 4j).

Folate analysis

We compared maternal liver and embryo folate profiles and total folates by a mass-spectrometry methodology that allows for analysis of unmetabolized folic acid and five major folate species, specifically dihydrofolate (DHF), tetrahydrofolate (THF), methylene-THF, 5-methyl-THF (5mTHF) and formyl-THF. Total folates and the proportion of folates in maternal liver did not differ significantly between treatment groups on the folate-deficient diet (Fig. 6a and b).

Differences were observed in the embryo folate profiles between the 1xDTG group and both control and 5xDTG groups. DHF and THF levels were significantly lower, and 5mTHF levels significantly higher, in the 1xDTG group (Fig. 6c), perhaps indicative of a methyl folate trap.[28] Embryo total folate levels did not differ significantly between the control and 5xDTG groups under folate-deficient conditions, although the 1xDTG group displayed higher total folate levels that did not reach significance ($p = 0.057$, by Kruskal–Wallis with Dunn's post-test) (Fig. 6d). We previously observed significantly higher foetal folate levels in the 5xDTG group as compared to control and 1xDTG groups under folate-replete conditions. This difference was not observed under folate-deficient conditions.

Discussion

Here we report findings from a large DTG-based ART fetotoxicity study in mice fed a folate-deficient diet. We compared our findings with previously published data from our lab on mice fed a folate-sufficient diet[18] to determine if there is an interaction between DTG and folate status that affects the risk of foetal anomalies. In this study mice were fed a folate-deficient diet for a minimum of 2 weeks prior to mating and continued on that diet throughout pregnancy. No NTDs were observed in the control arm and, with the exception of eye defects and bleeding defects which showed a non-significant increase in the control group under folate-deficient conditions, rates of all other defects were similar between folate-sufficient and folate-deficient conditions in the control group. This is expected as a folate-deficient diet alone has been shown to cause developmental delays but not specific anomalies.[12,29,30] This is in contrast to the DTG groups in the present study, where we observed several defects with significantly higher rates under folate-deficient conditions including NTDs, tail defects (indicative of spinal dysraphism), body asymmetry, abdominal wall defects, limb defects, and craniofacial defects. This suggests an interaction between DTG and maternal folate status that increases the risk for foetal defects.

In our previous study, under folate-sufficient conditions, we observed an unexpected non-monotonic response in foetal defects, with higher rates at the therapeutic DTG (1xDTG) dose compared with the control and supratherapeutic dose (5xDTG) groups, which had similar foetal defect rates.[18] Under folate-deficient conditions, however, not only did we observe an interaction between DTG and folate deficiency that led to higher rates of foetal defects, we also observed a dose–response with increasing DTG concentrations being associated with higher rates of foetal defects for most defects observed, although this is limited by the inclusion of only 2 doses of DTG and would require additional doses to determine definitively. This suggests that the protective effect observed previously under the higher 5xDTG dose required sufficient maternal folate levels. This is further supported by the higher foetal folate levels observed in the 5xDTG group under folate-sufficient conditions.

We observed a total of 16 foetuses with NTDs, which all occurred in the DTG groups (8 each in 1xDTG and 5xDTG), while no NTDs were observed in the control group. The rate of NTDs observed under folate-deficient conditions was higher than that under folate-sufficient conditions in both 1xDTG and 5xDTG. Our findings show that the risk for DTG-associated NTDs increases with folate deficiency in mice. This implies that sub-optimal folate status could also increase susceptibility to DTG-associated anomalies in humans and we speculate that such an interaction could explain why the initial NTD signal with

DTG use was observed in the Tsepamo study in Botswana, a country that does not have a folic acid fortification program.[2,3] Abdominal wall defects (omphalocele and gastroschisis) were also observed at a higher rate in pregnancies exposed to DTG from conception in the Tsepamo study,[3] as they were in our mouse study, and rates of these defects may similarly be reduced by folate supplementation.[31]

In vitro studies have suggested that DTG may be a partial antagonist of FOLR1,[15,16] and DTG treatment was associated with small changes in folate transporter expression and decreases in uptake.[17] The reports of small increases in serum folate levels in pregnant women taking DTG-based ART in the ADVANCE trial, may suggest that folate tissue distribution or folate transfer to the foetus could be affected.[14] In our study foetal folate levels did not differ significantly by DTG exposure. However, we did observe differences in folate proportions in the 1xDTG arm, which had lower proportions of DHF and THF and higher proportions of 5mTHF, suggesting that DTG may influence folate metabolism to produce a metabolic reaction previously called the 'methyl folate trap'.[28] This situation can arise if the methionine synthase mediated transfer of a methyl group from 5mTHF to homocysteine, which generates methionine and THF is impaired. Methionine synthase requires vitamin B12 and zinc as cofactors. DTG has been shown to have cation chelating properties and thus may interfere with this process by limiting access to zinc, although whether the degree of protein binding of drug influences its chelating properties remains to be determined.[32,33] These data would suggest that additional dietary factors could be considered in the context of DTG use in pregnancy, including vitamin B12 and zinc supplementation.[34] This may be particularly relevant to pregnant women with HIV, as vitamin B12 deficiency is thought to be more common in people living with HIV.[35] As zinc supplementation may influence the activity of DTG, it will be necessary to consider the timing of DTG and zinc intake.

Under folate-deficient conditions, in addition to NTDs, we also observed a severe left-right asymmetry phenotype that was significantly more common in the DTG-treated mice. These foetuses displayed whole-body torsion defects that were strongly asymmetric, as well as abdominal wall defects, limb defects, brain defects, and NTDs in some cases (2 exencephalies and 1 encephalocele in the 1xDTG group, 1 exencephaly and 1 encephalocele in the 5xDTG group). In a study using an *in vitro* pluripotent stem cell morphogenesis model, DTG treatment was associated with diminished axial elongation and altered expression of genes involved in embryonic patterning.[36] DTG effects on embryonic morphogenesis and axial patterning could have contributed to the severe left-right asymmetry defect we observed, although further studies are needed to confirm this.

Bleeding defects were also observed at a higher rate in the DTG arms versus control. These defects were significantly more frequent in the DTG groups under folate-deficient versus folate-sufficient conditions. Previous studies have demonstrated that high homocysteine levels are present in dams fed a folate-deficient diet.[12] If DTG alters methionine synthase activity, perhaps by interfering with the zinc cofactor, then homocysteine levels would be expected to increase even further and high homocysteine levels have been associated with increased risk for vascular disease.[37,38] Higher homocysteine levels have been reported in people living with HIV, although specific investigations in the context of DTG use are lacking.[35] DTG has also been shown to inhibit matrix metalloproteinases (MMPs),[39,40] which play an important role in a wide range of developmental processes including vascular remodelling, angiogenesis, and establishment of the blood–brain barrier.[41,42]

We observed no significant differences in foetal weight, placental weight, litter size, foetal viability, or resorption rates between control and DTG groups under folate-deficient conditions. However, DTG treatment was associated with lower crown-rump length as compared to control, suggesting that DTG may impact foetal body length, at least under folate-deficient conditions. We also observed an almost 3-fold increase in the rate of growth retarded foetuses in both DTG groups versus control. Growth retardation did not differ significantly between groups under folate-sufficient conditions, but was higher in the DTG groups under folate-deficient conditions.

Our study has the advantage of large size and inclusion of a concurrent non-DTG control group. Further, by keeping our study design the same as in our previous study of folate-sufficient conditions, we are able to make comparisons based on folate status. Our study also has several limitations, including the evaluation of only external anomalies in our analysis, and inclusion of only two dosages of DTG limiting our ability to accurately assess a dose–response relationship. Additionally, while our use of DTG in a clinically relevant triple–therapy combination better models the clinical scenario, it does make it difficult to separate DTG effects from those of the NRTI backbone. However, the inclusion of the 5xDTG group, where the DTG dose was increased while the NRTI dose remained the same, allowed us to observe differences related specifically to DTG. Finally, while the median time on the folate-deficient diet was the same between groups, time on the folate-deficient diet did vary between dams, depending on how long it took for mice to get plugged. We explored whether this could have influenced defect rates but did not observe any associations between time on the diet and presence of any of the defects observed.

In conclusion, our data show an interaction between DTG treatment and folate-deficient diet that is

associated with increased incidence of NTDs and several other foetal anomalies including abdominal wall, and bleeding defects. We also observed foetuses with a severe left-right asymmetry phenotype, a defect not seen under folate-sufficient conditions, which suggests that DTG could influence axial patterning in development, although this has not been seen clinically. While the original NTD signal reported for DTG in the Tsepamo study has now largely waned, continued surveillance for defects particularly those related to anterior body wall closure, axial symmetry, or vascular integrity may be merited. Our data suggest a relationship between DTG, maternal folate intake and foetal defects and, hence, support the use of folic acid supplementation in the context of DTG treatment in pregnancy. Additionally, proper nutrition including B12 and zinc supplementation are worth considering given the alterations in foetal folate profile we observed in the 1xDTG group. Establishing an adequate folate, vitamin B12, and zinc status in women of childbearing age who are taking DTG is likely to be important to both help avoid NTDs and other birth defects, and to counteract the potential for high homocysteine levels and possible associated maternal conditions, including gestational hypertension.[43,44] This is be particularly important in areas where folate fortification programs are not in place. A temporal gap between dosing of DTG and zinc should be considered to avoid potential interactions that could influence DTG antiviral activity.

Contributors

HM performed all breeding and foetal assessment experiments with help from JN, AY, EYL, TS, and OT. BM and HM performed all the facial morphometry assessments. KL performed the mass spectrometry experiments under the direction of NDEG. CG and TDY performed the magnetic resonance experiments under the direction of JGS. LS and AJC performed the blinded foetal assessments, with input from NDEG. LS, HM, and VD performed all foetal anomaly analyses/interpretation. LS conceived the study and directed the research, with input from AJC and NDEG. HM and LS drafted the manuscript with major contributions from AJC, NDEG, and JGS. The corresponding (LS) and first (HM) authors had full access to and verified all the data in the study. The corresponding author (LS) had final responsibility for the decision to submit for publication. All authors read and approved the final version of the manuscript.

Data sharing statement

All relevant data have been presented in the manuscript. All requests for or questions about the data can be initiated by contacting lena.serghides@utoronto.ca.

Declaration of interests

The authors have no conflicts of interest relating to this study.

Acknowledgements

We thank the technicians and vets in the University Health Network Animal Care Facility for their assistance with the care of animals. We thank Westat for project management. This project has been funded by Federal funds from the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Department of Health and Human Services, under Contract No. HHSN275201800001I and award #R01HD104553. We acknowledge the support of the National Institute for Health Research Biomedical Research Centre at Great Ormond Street Hospital for Children NHS Foundation Trust and University College London. We are grateful for the support and assistance of the UCL Institute of Child Health Biological Mass Spectrometry facility. LS is supported by a Tier 1 Canada Research Chair in Maternal-Child Health and HIV. HM is supported by a Junior Investigator award from the Ontario HIV Treatment Network.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2023.104762.

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