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The association between prenatal alcohol consumption and preschool child stress system disturbance

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Abstract

Background: Drinking alcohol during pregnancy is considered a risk factor for child development; however, child biomarkers of prenatal alcohol exposure have been rarely studied. We examined whether a meconium alcohol metabolite (ethyl glucuronide, EtG) was associated with child cortisol concentrations at primary school age.

Methods: For 137 children, prenatal alcohol exposure was operationalized by the meconium biomarker EtG and by maternal self-reports during pregnancy. Two EtG cut-offs (EtG ≥10 ng/g and EtG ≥112 ng/g) were applied. Cortisol concentrations were measured in saliva and hair samples.

Results: Children with EtG ≥10 ng/g showed significantly reduced hair cortisol concentrations (HCCs) ($p = .050$, $\eta_p^2 = 0.042$). For children with EtG ≥112 ng/g, the cortisol awakening response (CAR) was significantly decreased ($p = .025$, $\eta_p^2 = 0.070$). These effects were also present in correlational analyses with continuous EtG data, speaking for partly dose-dependent effects. Especially, within the EtG ≥112 ng/g group, the basal (CAR: $r_p = -.642$, $p = .120$) and cumulative (HCC: $r_p = -.660$, $p = .107$) cortisol parameters were associated with child emotional symptoms at medium effect size.

Conclusions: The present study showed both the biological association of intrauterine alcohol exposure with the cortisol stress system, partly dose-dependent, and the functional association with emotional and behavioral symptoms.

KEYWORDS

alcohol, biomarker, child, cortisol stress system, ethyl glucuronide, hypothalamus-pituitary-adrenal axis, intrauterine alcohol exposure, meconium, pregnancy

1 | INTRODUCTION

Alcohol consumption during pregnancy is one of the most verified prenatal risk factors for child development. Nonetheless, about 10% of pregnant women report drinking alcohol during pregnancy worldwide. The European prevalence is even higher (Lange et al., 2017). It is well known that alcohol consumption during pregnancy and the subsequent intrauterine exposure can cause late presentations of

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alcohol-related developmental abnormalities. Current research addresses the underlying mechanisms mediating alcohol exposure and developmental disturbances. While there are many ways to verify the real prenatal alcohol exposure and to show developmental consequences, research is still needed to clarify many of these pathways to fully understand the underlying mechanisms in order to develop proper diagnostics. Until now, most studies examine the relation of prenatal alcohol consumption with neuropsychological outcomes, that is cognitive and behavioral impairments (Comasco, Rangmar, Eriksson, & Oreland, 2018; Flak et al., 2014). Studies, which describe neurobiological associations, are rarer. The hypothalamic–pituitary–adrenocortical (HPA) axis, a major neuroendocrine system regulating diverse physiological functions, is supposed to be one mediating mechanism of prenatal stress to child psychopathology (Rakers et al., 2017). This system has been shown to be perturbed after prenatal alcohol exposure, while most studies found elevated cortisol levels during the day (Keiver, Bertram, Orr, & Clarren, 2015; McLachlan et al., 2016). A meta-analysis published in 2015 revealed greater effect sizes for alcohol use—compared to other prenatal risks—and measures of basal cortisol levels—compared to reactivity measures (Pearson, Tarabulsy, & Bussieres, 2015) in children before 60 month of age.

All of these studies rely on maternal self-reports or expert fetal alcohol syndrome diagnosis to categorize prenatal alcohol exposure versus controls. However, earlier works have shown that there is little correspondence between maternal self-reports during pregnancy and biomarker results of intrauterine alcohol exposure (Eichler et al., 2016; Gomez-Roig et al., 2018; Lamy et al., 2017). In a random sample from the general population, there were about 15% of ethyl glucuronide (EtG) levels over the threshold, while no mother reported heavy alcohol consumption (Abernethy et al., 2018). A meta-analysis of Lange et al. found the prevalence of prenatal alcohol consumption measured in newborn's meconium to be 4.26 times higher than the prevalence measured by maternal self-reports (Lange, Shield, Koren, Rehm, & Popova, 2014). Concerning child development after prenatal alcohol exposure, the predictive value of a meconium biomarker is higher than of the maternal self-report (Eichler et al., 2018).

It seems worthwhile to establish these biomarkers of intrauterine alcohol exposure as valid predictors of child development. Measurement of ethanol biomarkers in newborn meconium is a feasible way to determine the prevalence of alcohol consumption in pregnancy. One of these markers is EtG, a minor ethanol metabolite. It can be detected in the first stool (meconium) of the newborn, passed within 72 hr after birth. Meconium accumulates in the fetal gut from around the 20th week of gestation until birth. The majority of the meconium (75%) is created during the last 8 weeks of pregnancy. Positive cut-off for intrauterine alcohol exposure varies from study to study; in most studies, a minimum of 10 ng/g EtG (equivalent to the limit of detection) argues for fetal alcohol exposure during the third trimester (Bakdash et al., 2010; Eichler et al., 2018; Frey et al., 2018; Himes et al., 2015). Himes et al. (2015) point out that searching for the most appropriate cut-off for intrauterine alcohol exposure still needs studies which validate meconium EtG cut-offs against reliable self-report measures. However, EtG was found to be applicable for detection of even low levels of alcohol exposure (Bager, Christensen, Husby, & Bjerregaard, 2017). To examine the validity of EtG as a biomarker for intrauterine alcohol exposure, it is essential to check the correspondence with varying child developmental outcomes. Until now, there are only two studies which have found a correspondence between meconium EtG levels and impaired child brain development: meconium EtG levels were associated with adverse cognitive outcomes (Eichler et al., 2018) and distinct whole-genome DNA methylation patterns in primary school children (Frey et al., 2018). Studies associating the meconium biomarkers with child neurobiological outcomes, that is HPA axis activity, are entirely missing.

Earlier studies have shown that prenatal alcohol exposure correlates with HPA dysregulation throughout life, mostly marked by hypercortisolism and greater activation of the HPA system in stress situations—even for low levels of alcohol consumption (Haley, Handmaker, & Lowe, 2006; Ouellet-Morin et al., 2011). Until now, all studies, which examined altered cortisol system activities after intrauterine alcohol exposure, were based on maternal self-reports and mostly performed with toddlers (Hellemans, Sliwowska, Verma, & Weinberg, 2010). Taking the questionable validity of maternal self-reports into account, studies based on biomarkers of prenatal alcohol exposure are missing. In the present study, we examined the association of meconium EtG with preschooler's (6–9 years of age) basal cortisol activity for the first time. Correlations between continuous EtG levels and basal cortisol markers are presented. We hypothesized that children with a positive meconium EtG (≥10 ng/g and a "higher-risk level" of ≥112 ng/g, respectively) show altered basal cortisol levels during the day (salivary specimens) and altered cumulative cortisol levels during the last month (hair specimens). To test the potential benefits of the meconium EtG in comparison to maternal self-reports and to validate meconium EtG cut-offs against self-report measures, we tested whether the significant differences obtained in the EtG positive (EtG+) versus EtG negative (EtG–) groups could also be found on the basis of maternal self-reports. While a comprehensive validation should stem from a combination of results associating biomarker levels with child outcomes in different developmental areas—this study is intended to contribute to the establishment of meconium EtG as a biomarker of prenatal alcohol consumption in association with HPA axis disturbance.

2 | PATIENTS AND METHODS

2.1 | Study design

The present study included two assessment times: for the first assessment, pregnant women were recruited to take part in FRAMES (**Fra**nconian **M**aternal Health **E**valuation **S**tudies) (Reulbach et al., 2009) from the outpatients at the Department of Obstetrics and Gynecology in Erlangen, Germany. Women were in their third trimester with a minimum age of 18 years old and 30 weeks gestation time. Child meconium was collected after birth (Bakdash et al., 2010). The second assessment took place at the Department

of Child and Adolescent Mental Health in Erlangen, Germany, when the children were 6 to 9 years old. In this follow-up study (FRANCES; **Fran**conian **C**ognition and **E**motion **S**tudies) (Eichler et al., 2016), multiple assessments were conducted with the child and his/her mother, including basal salivary and cumulative hair cortisol, maternal psychopathology, and information on socioeconomic variables. The study was approved by the local Ethics Committee and was conducted in accordance with the Declaration of Helsinki. All parents and children gave informed consent/assent.

2.2 | Sample characteristics

There were a total of 147 children with EtG measures, who gave saliva and hair samples. Children were completely excluded from analyses when there was an endocrine disease ($n = 2$, Henoch-Schönlein purpura) or a medication with corticosteroids ($n = 6$) or ketoconazole ($n = 1$) within the last half year. Other relevant diseases or medications were not present in the sample. For saliva analyses, one child was further excluded because of collecting the samples on different days. Consequently, the present paper reports the data of 137 children. Sample characteristics are reported in Table 1. Of the 137 children, there were 36 children (26.3%) with a meconium EtG ≥10 ng/g ($EtG_{10}+$) and 22 children with a meconium EtG ≥112 ng/g ($EtG_{112}+$, 16.1%). Their outcomes were compared to non-exposed controls (EtG negative, $EtG_{10}-$ $n = 101$, $EtG_{112}-$ $n = 115$). For maternal self-reports, there were 35 mothers (25.5%) who reported alcohol consumption during pregnancy. The correspondence between maternal self-reports and child meconium EtG was small in the cohort (≥10 ng/g cut-off: $\varphi = 0.15$, $p = .09$; ≥112 ng/g cut-off: $\varphi = 0.06$, $p = .46$).

2.3 | Instruments and measures

2.3.1 | Prenatal alcohol consumption

2.3.1.1 | Meconium Ethylglucoronide

Meconium EtG is a minor ethanol metabolite, which accumulates in the fetal gut from around the 20th week of gestation until birth and can be detected in the meconium of the newborn as a third trimester marker of intrauterine alcohol exposure (Cabarcos et al., 2014; Himes et al., 2015). One gram of meconium was collected from the newborns within the first 2–24 hr after birth. EtG was determined as described in detail by Bakdash et al. (2010). The positive EtG cut-off for intrauterine

TABLE 1 Frequency, means, and standard deviations of sample characteristics

	Total	$EtG_{10}-$	$EtG_{10}+$	Statistics		$EtG_{112}-$	$EtG_{112}+$	Statistics	
	137	101	36	χ^2/t	p	115	22	χ^2/t	p
Self-report (yes)	35	22	13	2.87	.091$^+$	28	7	0.54	.462
Sex (male/female)	66/71	48/53	18/18	0.07	.799	55/60	11/11	0.04	.852
Child age (years)	7.51 (0.60)	7.42 (0.54)	7.78 (0.69)	−3.14	.002**	7.46 (0.60)	7.82 (0.54)	−2.65	.009**
Apgar	9.48 (0.50)	9.50 (0.47)	9.43 (0.57)	0.76	.448	9.49 (0.52)	9.44 (0.38)	0.39	.695
Birth weight (gram)	3,444 (486)	3,376 (442)	3,634 (557)	−2.80	.006**	3,401 (477)	3,665 (481)	−2.37	.019*
Maternal age at birth (years)	32.8 (4.76)	32.8 (5.01)	33.1 (4.01)	−0.34	.738	32.9 (4.81)	32.7 (4.57)	0.13	.899
Socioeconomic status (index)	11.3 (2.16)	11.1 (2.23)	11.6 (1.93)	−1.15	.252	11.2 (2.18)	11.7 (2.08)	−1.01	.315
Child total psychopathology	7.84 (4.94)	7.57 (4.92)	8.58 (4.96)	−1.05	.294	7.65 (4.77)	8.82 (5.73)	−1.02	.312
Child emotional problems (internalizing)	1.81 (1.69)	1.74 (1.70)	2.00 (1.67)	−0.78	.435	1.70 (1.64)	2.36 (1.87)	−1.69	.094$^+$
Child conduct problems (externalizing)	1.99 (1.66)	1.90 (1.56)	2.22 (1.91)	−0.91	.369	1.97 (1.62)	2.09 (1.88)	−0.33	.746
Maternal psychopathology	47.7 (12.9)	46.0 (12.1)	52.2 (14.2)	−2.35	.023*	46.3 (12.7)	54.4 (12.0)	−2.75	.007**

Note: Socioeconomic status: combination of maternal/paternal education level (four levels: <9, 9, 10, or 13 years) and net family income (six levels: <1,000 to >5,000) (sum-index, theoretical range: 3–14). Apgar score: best adaption = 10. Total child psychopathology/emotional and conduct problems: Strength and Difficulties Questionnaire (Goodman, 2001). Maternal psychopathology: Brief Symptom Inventory (Franke, 2000). χ^2: $df = 1$; t: $df = 133$–135.

Abbreviation: EtG, ethyl glucuronide.

$^+p < .10$,

$^*p < .05$,

$^{**}p < .01$.

alcohol exposure varies slightly from study to study. A minimum of 10 ng/g EtG argues for fetal alcohol exposure. In the present study, the first cut-off was set at 10 ng/g EtG (EtG_{10}− $n = 101$, EtG_{10}+ $n = 36$). Other studies chose secondary higher cut-offs (Goecke et al., 2014; Himes et al., 2015) and found additional effects. Therefore, in the present study, a second cut-off was set. We applied a 112 ng/g cut-off, which guaranteed a minimum of 10 children in each cell of the multivariate tests (EtG_{112}− $n = 115$, EtG_{112}+ $n = 22$).Maternal self-report during pregnancy was examined in a structured interview. Women in their third trimester were questioned about their drinking behavior during pregnancy (No, I do not drink in general; No, I did not drink during pregnancy; Yes, I drank rarely during pregnancy; Yes, I drank 1 glass/day during pregnancy; Yes, I drank more than 1 glass day during pregnancy). For data analysis, two groups were created based on women's self-reports: no drinking ("I don't drink in general" + "I didn't drink during pregnancy") versus drinking ("I rarely drank during pregnancy" + "I drank one glass/day during pregnancy").

2.3.2 | Basal cortisol activity

Mothers were instructed to collect five saliva samples at home over a 24-hr period from the study child (Sarstedt, Salivette®): (*t*1) immediately after awakening, (*t*2) 30 min later, (*t*3) 12:00 a.m., (*t*4) 5:00 p.m., and (*t*5) at bedtime. Restrictions were to not eat, drink, or brushing their teeth 30 min before sampling. Parents additionally filled out a protocol during the sampling day, reporting on sleeping times, sport activities, medication, or other particular events and were informed that sampling should not be performed in case of acute sickness, injuries, or inflammation in the mouth. Analyses were performed in the neurobiological laboratory of the Department of Child and Adolescent Mental Health, University Hospital Erlangen. Saliva samples were centrifuged at +4°C (1,000*g*, 2 min) and stored at −20°C. Cortisol levels were analyzed with a luminescence immunoassay (ELISA; IBL International, RE62111/RE62119). Photometric measurements were conducted with the Multiskan™ GO microplate spectrophotometer (Thermo Fisher Scientific). Due to the typical positive skew of cortisol data, natural logarithm transformation (ln) was employed to the raw sample data to improve normal distribution (Adam & Kumari, 2009). Five parameters were calculated out of the ln-transformed raw values: wakening cortisol (WakeC, value at *t*1), cortisol awakening response (CAR, increase from *t*1 to *t*2: *t*2–*t*1), bedtime cortisol (BedC, value at *t*5), diurnal cortisol slope (SlopeC, slope over *t*1, *t*3, *t*4, *t*5; *t*2 excluded), and daytime cortisol (DayC, area under the curve with respect to ground, AUCg) (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003).

After ln-transformation, all single saliva cortisol measures were within a 3 *SD* range from the mean. For saliva samples, time frames were set for the first two samples in order to assess the sensitive cortisol reaction in the morning accurately: for the first assessment, samples that were collected more than 15 min after awakening were excluded from analyses of WakeC, CAR, and SlopeC ($n = 27$). If the second sample was collected less than 15 min or more than 45 min after awakening, it was excluded from CAR calculation ($n = 44$). Because of missing values in awakening time, resulting in unknown time frames for the morning, 16 children were further excluded from WakeC, CAR, and SlopeC analyses. The DayC ($n = 1$) and the SlopeC ($n = 1$) were not calculated, when there were fewer than three valid cortisol values to be included. Therefore, the analyzed groups vary from test to test: WakeC $n = 90$, CAR $n = 75$, BedC $n = 133$, SlopeC $n = 90$, and DayC $n = 133$ (Table 2).

2.3.3 | Cumulative cortisol level

The child's hair cortisol concentrations (HCCs) were measured from the scalp near hair segment (1 cm), representing hair growth over a period of approximately 1 month. Mothers were asked about the chemical treatment of their children's hair: no child had dyed or bleached hair. Child body mass index (BMI) was determined and there were no obesity scores (BMI >30 kg/m^2). For protein extraction, an incubation step with 2-mL methanol for 24 hr was performed. The protein extract was further evaporated at 60°C overnight and dissolved in *phosphate-buffered saline*. Samples were stored at +4°C. The total amount of protein was measured using the *Bradford Protein Assay*. Cortisol concentrations were measured using a specific salivary cortisol ELISA kit from Abnova Corporation (KA1885). This is a solid-phase ELISA using a polyclonal rabbit antibody directed against cortisol. The assay is based on the principle of competitive binding, and endogenous cortisol in the sample competes with a cortisol–horseradish peroxidase conjugate for binding to the antibody. This ELISA has an intra-assay variability of 8.27% and inter-assay variability of 8.33% with a spiking recovery of 100% and calibration range of 0.28–82.8 nmol/L. The raw values were transformed as followed: $\mathrm{HCC} = \ln\left(\frac{\text{cortisol concentration ng/ml}}{\text{total protein concentration ng/ml}} \times 10^{-6}\right)$. For hair analyses, in 29% of cases ($n = 40$), the collected amount of hair was not enough to determine the total protein and cortisol concentrations. Two ln-transformed "hair cortisol to total protein ratios" were excluded, because they fell out of the 3 *SD* range. Therefore, the analyzed group for HCC was 96 (Table 2).

2.3.4 | Child psychopathology

Child psychopathology was assessed by the *Strength and Difficulties Questionnaire* (SDQ) (Goodman, 2001; Klasen, Woerner, Rothenberger, & Goodman, 2003). A total of 25 items were rated for relevancy to their child by the mother (0 = not relevant, 1 = partly relevant, 2 = totally relevant). The total score and the subscales *Emotional Problems* (5 items) and *Conduct Problems* (5 items) were used as indices for child's psychopathology.

2.4 | Confounder

We tested the following variables as potential confounders for predicting child cortisol concentrations in hair and saliva: sex,

TABLE 2 Testing potential confounders: Pearson's correlations with child cortisol parameters (*r* [*p*])

	WakeC	CAR	DayC	SlopeC	BedC	HCC
	n = 90	*n* = 75	*n* = 133	*n* = 90	*n* = 133	*n* = 96
Sex	−0.045 (0.672)	−0.080 (0.494)	−0.100 (0.253)	−0.002 (0.982)	−0.111 (0.202)	−1.20 (0.244)
Child age	0.005 (0.962)	0.060 (0.607)	0.044 (0.617)	−0.121 (0.258)	−0.027 (0.758)	0.022 (0.833)
Apgar	0.161 (0.134)	−0.027 (0.821)	−0.069 (0.434)	−0.256* (0.016)	−0.235** (0.007)	0.066 (0.526)
Birth weight	−0.120 (0.261)	0.049 (0.674)	0.074 (0.398)	0.128 (0.228)	0.067 (0.444)	−0.057 (0.582)
Socioeconomic status	0.132 (0.215)	0.061 (0.603)	0.085 (0.331)	−0.086 (0.423)	−0.008 (0.924)	−0.029 (0.782)
Child total psychopathology	0.131 (0.218)	−0.072 (0.539)	0.037 (0.671)	−0.042 (0.691)	0.101 (0.247)	0.092 (0.372)
Child emotional problems	0.013 (0.904)	−0.168 (0.149)	−0.070 (0.425)	−0.012 (0.908)	0.036 (0.679)	−0.080 (0.440)
Child conduct problems	0.170 (0.110)	−0.070 (0.552)	0.047 (0.594)	−0.043 (0.684)	0.135 (0.122)	0.014 (0.889)
Maternal psychopathology	−0.087 (0.414)	−0.088 (0.945)	0.123 (0.160)	0.074 (0.487)	0.080 (0.363)	−0.108 (0.299)
School-day yes/no	−0.153 (0.149)	0.039 (0.738)	−0.305** (0.000)	0.122 (0.252)	0.075 (0.391)	-
Time *t*1–*t*5	0.017 (0.874)	0.170 (0.145)	0.648** (0.000)	0.081 (0.449)	−0.259** (0.003)	-
Time wakening to *t*1	−0.06 (0.574)	0.12 (0.318)	−0.12 (0.155)	0.00 (0.978)	0.12 (0.172)	-
Antibiotic intake	0.203+ (0.054)	−0.051 (0.661)	−0.005 (0.957)	−0.217* (0.040)	−0.140 (0.107)	0.069 (0.502)

Note: Socioeconomic status: combination of maternal/paternal education level (four levels: <9, 9, 10, or 13 years) and net family income (six levels: <1,000 to >5,000) (sum-index, theoretical range: 3–14). Apgar score: best adaption = 10. Total child psychopathology/emotional and conduct problems: Strength and Difficulties Questionnaire (Goodman, 2001); Maternal psychopathology: Brief Symptom Inventory (Franke, 2000). χ^2: *df* = 1; *t*: *df* = 133–135. (*t*1) immediately after awakening, (*t*2) 30 min later, (*t*3) 12:00 a.m., (*t*4) 5:00 p.m., and (*t*5) at bedtime. Sex: (1 = male, 2 = female). Antibiotic intake: (yes = 1/ no = 0).

Abbreviations: BedC, bedtime cortisol; CAR, cortisol awakening response; DayC, daytime cortisol; HCC, hair cortisol concentration; SlopeC, daily slope; WakeC, wakening cortisol.

+*p* < .10,

**p* < .05,

***p* < .01.

child age, Apgar score, birth weight, socioeconomic status, child psychopathology, maternal psychopathology, and antibiotic intake. Additional confounders were considered regarding saliva cortisol concentrations: school-day (yes/no), time "*t*1 to *t*5", and time "wakening to *t*1". Immediately after delivery, birth weight (gram) and Apgar scores (maximum 10) were registered in the maternity room. The socioeconomic status was calculated by maternal and paternal secondary education level and net family income. A socioeconomic status sum index was created (theoretical range: 3–14). Maternal actual psychopathology was assessed with the *Brief Symptom Inventory* (BSI) (Franke, 2000), a 53 items (5-point Likert) self-rating questionnaire related to mental stress during the last 7 days. A summed global index (global severity index, GSI, T-score) was used.

Due to the significant differences in child age, birth weight, and current maternal psychopathology between the EtG+ and the EtG− groups in our sample (Table 1), we controlled these variables in all analyses. Additionally, because of significant correlations (Table 2), the following variables were controlled in specific analyses: the Apgar score for prediction of SlopeC and BedC; school-day yes/no for the DayC; time from *t*1 to *t*5 for the DayC and the BedC; antibiotic intake for the WakeC and the SlopeC.

2.5 | Statistical analysis

The analyses were carried out using the IBM SPSS Statistics (Version 21.0, IBM Corp, 2012). Descriptive data were reported as means (*M*) and standard deviations (*SD*). Significance was tested using the *t*-test. Associations between the continuous EtG levels and basal cortisol markers were tested in partial correlations (confounder-controlled). Log-transformed ($\log_{10}$) EtG values were considered for correlational analyses because the continuous EtG values were not normally distributed (total sample: Shapiro-Wilk *W*[137] = 0.349, *p* = .000; EtG_{10}+: Shapiro-Wilk *W*[36] = 0.661, *p* = .000; EtG_{112}+: Shapiro-Wilk *W*[22] = 0.724, *p* = .000). Homogeneity of variance in the categorical EtG versus control groups was tested using the Levene's test. Only in one case (BedC in ≥10 ng/g versus controls) variances were not homogeneous. Participant EtG levels ≥10 ng/g versus <10 ng/g and ≥112 ng/g versus <112 ng/g, respectively, were used as the between-subject factor in separate ANCOVAs. Confounders were controlled as covariates. If there was a significant association of EtG level and child cortisol measure, the same analysis was run for self-reported alcohol consumption (yes/no) as the between-subject factor. Partial eta-squared (η_p^2, ANCOVA) and Cohen's *d* (*t*-test) were reported as the effect size measures. According to

TABLE 3 Cortisol levels and diurnal cortisol parameters in the total sample and in EtG− versus EtG+ groups (*t*-tested) and partial correlations with continuous EtG levels

	Total		EtG_{corr}	$EtG_{10+corr}$	$EtG_{112+corr}$	EtG_{10}−		EtG_{10}+						EtG_{112}−		EtG_{112}+				
	n	*M* (*SD*)	r_p (*p*)	r_p (*p*)	r_p (*p*)	*n*	*M* (*SD*)	*n*	*M* (*SD*)	*df*	*t*	*p*	*d*	*n*	*M* (*SD*)	*n*	*M* (*SD*)	*df*	*t*	*p*	*d*
Diurnal cortisol levels (nmol/L)																					
*t*1	132	15.9 (8.29)	.069 (.465)	.013 (.950)	.319 (.213)	98	15.7 (8.24)	34	16.3 (8.56)	130	0.36	.719	0.07	111	15.9 (8.19)	21	15.9 (9.01)	130	0.01	.989	0.00
*t*2	135	17.8 (8.28)	−.193 (.040)*	−.191 (.340)	−.036 (.892)	99	18.4 (8.51)	36	16.1 (7.49)	133	1.38	.169	0.28	113	18.5 (8.47)	22	14.1 (6.20)	133	2.28	.024*	0.54
*t*3	131	5.61 (2.32)	−.118 (.210)	−.087 (.667)	.445 (.074)+	97	5.59 (2.42)	34	5.66 (2.04)	129	0.16	.874	0.03	110	5.70 (2.39)	21	5.13 (1.92)	129	1.02	.309	0.25
*t*4	131	3.83 (1.67)	.012 (.896)	−.072 (.721)	.477 (.053)+	98	3.83 (1.63)	33	3.84 (1.81)	129	0.02	.983	0.01	110	3.90 (1.66)	21	3.48 (1.69)	129	1.06	.293	0.25
*t*5	134	1.86 (1.04)	−.105 (.268)	.210 (.294)	.513 (.035)*	98	1.93 (1.13)	36	1.67 (0.69)	132	1.32	.188	0.25	112	1.89 (1.09)	22	1.72 (0.73)	132	0.72	.476	0.16
Diurnal cortisol parameters (nmol/L)[a]																					
WakeC	90	2.76 (0.55)	.041 (.710)	.242 (.277)	.405 (.170)	64	2.76 (0.54)	26	2.74 (0.60)	88	0.18	.854	0.04	73	2.76 (0.52)	17	2.72 (0.68)	88	0.27	.786	0.07
CAR	75	0.05 (0.17)	−.230 (.052)+	−.536 (.032)*	−.631 (.129)	56	0.07 (0.18)	19	0.02 (0.15)	73	1.14	.258	0.29	111	0.07 (0.17)	10	−0.04 (0.16)	73	1.91	.060+	0.65
BedC	133	0.99 (0.32)	−.129 (.153)	.156 (.409)	.444 (.085)+	97	1.00 (0.34)	36	0.95 (0.26)	131	0.98	.329	0.16	65	0.99 (0.33)	22	0.97 (0.25)	131	0.32	.748	0.06
SlopeC	90	−0.13 (0.05)	−.145 (.190)	−.030 (.902)	−.153 (.653)	64	−0.13 (0.05)	26	−0.14 (0.04)	88	1.16	.249	0.21	73	−0.13 (0.05)	17	−0.14 (0.05)	88	0.67	.508	0.20
DayC	133	23.4 (3.80)	−.008 (.933)	.004 (.984)	.502 (.040)*	97	23.6 (3.75)	36	22.9 (3.92)	131	0.88	.383	0.18	111	23.6 (3.66)	22	22.2 (4.34)	131	1.54	.126	0.37
Cumulative cortisol level (ratio)[b]																					
HCC	96	3.84 (1.11)	−.209 (.045)*	−.160 (.554)	.200 (.668)	77	3.96 (1.11)	19	3.34 (0.96)	94	2.24	.027*	0.57	86	3.92 (1.08)	10	3.12 (1.09)	94	2.20	.030*	0.74

Note: EtG_{corr} = partial correlations r_p of $\log_{10}(x + 1)$ transformed continuous EtG data: all correlations were controlled for child age, maternal psychopathology and birth weight. Additionally controlled for WakeC: antibiotics intake in the 6 months before sample collection, for BedC: time between first and last sample, mean Apgar score, for SlopeC: antibiotics intake in the 6 months before sample collection, mean Apgar score, for DayC: time between first and last sample, school-day yes/no. Sampling times: *t*1 = at awakening, *t*2 = 30 min after awakening, *t*3 = 12 a.m., *t*4 = 5 p.m., *t*5 at bedtime. Exclusion of participants due to diseases, medications, and technical problems. Exclusion of *T*1 samples with >15 min since awakening or *T*2 samples with <15 min or >45 min since awakening as well as missing values in awakening time. *t*-statistics and *p*-values refer to the independent *t*-test with *t*-scores displayed as absolute values.

Abbreviations: BedC, bedtime cortisol; CAR, cortisol awakening response; DayC, daytime cortisol; DayC, total cortisol release throughout the day; EtG, ethyl glucuronide; HCC, hair cortisol concentration; SlopeC, daily slope; WakeC, wakening cortisol.

[a]Based on ln-transformed raw cortisol values.

[b]Based on ln-transformed "hair cortisol to total protein ratio * 10^6".

+$p < .10$,

*$p < .05$,

Cohen (1988), $0.01 \le \eta_p^2 \le 0.05$ and $0.20 \le d \le 0.49$ were interpreted as a small effect, $0.06 \le \eta_p^2 \le 0.13$ and $0.50 \le d \le 0.79$ as a medium effect, and $\eta_p^2 > 0.13$ and $d > 0.79$ as a large effect. Because of the exploratory character of the study, we did not correct for multiple testing. The level of significance was defined as $p < .05$ (two-tailed).

3 | RESULTS

Table 3 reports the descriptive data for the five saliva cortisol measures during the day, the diurnal cortisol parameters, and the HCC separated for EtG+ and EtG− children along the two cut-offs. Figure 1 describes the saliva cortisol concentrations over the five sampling times and Figure 2 shows the HCC distribution in the groups. There were 36 (25.5%) women with an EtG+ child (EtG level within the EtG_{10}+ group: $M = 374.4$, $SD = 532.9$, range 10.0–2,400.0; EtG level within the EtG_{112}+ group: $M = 577.5$, $SD = 601.2$, range 142.0–2,400.0). In the total sample, there were 102 women (74.5%) who reported no alcohol consumption during pregnancy and 35 (25.5%) women who reported alcohol consumption during pregnancy. The correlation between maternal drinking self-report and meconium EtG biomarker was non-significant with $\varphi = 0.145$, $p = .091$ (EtG ≥10 ng/g) and $\varphi = 0.063$, $p = .462$ (EtG ≥112 ng/g), respectively.

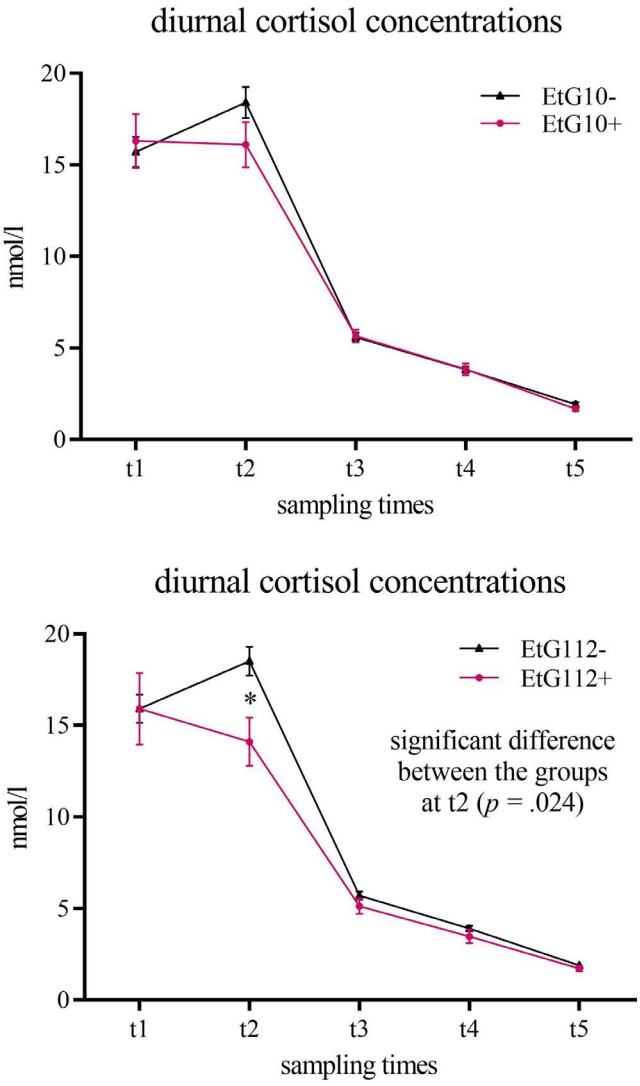

FIGURE 1 Cortisol concentrations over the day for the EtG ≥10 ng/g (EtG_{10}+ $n = 36$) and the ≥112 ng/g (EtG_{112}+ $n = 22$) groups (with error bars). * $p < .05$

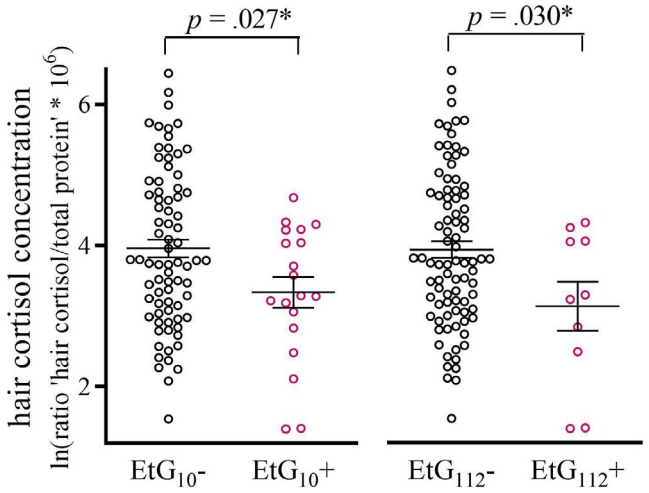

FIGURE 2 Hair cortisol concentrations for the EtG ≥10 ng/g (EtG_{10}+ $n = 19$) and the ≥112 ng/g (EtG_{112}+ $n = 10$) groups (with error bars)

Speaking for the existence of a dose-response effect, the continuous EtG levels were partly correlated with the cortisol outcomes (confounder-controlled, see Table 3): the more EtG in newborn meconium, the greater the cortisol activity impairment. This effect—with low to high effect size measures (overall group: $r_p = -.230$, EtG_{10}+ and EtG_{112}+ group: $r_p = -.536$, $r_p = -0.631$) and a significant result for the EtG_{10}+ group—was found for the CAR with most consistency.

Table 4 reports the ANCOVA results. For the 10 ng/g cut-off, there were no significant differences in the diurnal cortisol parameters between the EtG+ and the EtG− groups. However, affected children showed significantly lower HCC values (Figure 2) (non-significant for maternal self-report: $F(1, 90) = 0.065$, $p = .799$). For the 112 ng/g cut-off, there was a significantly different CAR of medium effect size (non-significant for maternal self-report: $F(1, 70) = 0.230$, $p = .633$), which was additionally reflected in a significant lower cortisol level at *t*2 (Table 3) (non-significant for maternal self-report: $F(1, 129) = 0.092$, $p = .762$).

The CAR analysis (Figure 1) revealed a missing increase from *t*1 to *t*2 in the EtG_{112}+ group. However, the CAR, which represents the difference between the WakeC (*t*1) and the *t*2 cortisol (30 min after *t*1), is sensitive to violations of sampling time. Table 5 shows small effect size differences between the EtG_{112}− and the EtG_{112}+ groups in the CAR-related parameters, that is a later wakening and *t*1 and *t*2 sampling. Time windows (wakening to *t*1 and *t*1–*t*2) were not affected. A theory-contrary negative CAR (*t*2 minus *t*1 cortisol) was not significantly correlated with maternal ($r = .104$, $p = .239$) or child (total: $r = -.012$, $p = .892$; emotional: $r = -.096$, $p = .275$; conduct: $r = -.020$, $p = .822$)

TABLE 4 EtG-associated differences in diurnal cortisol parameters: ANCOVA results

	n		*F*	*p*	η_p^2
	EtG_{10}−	EtG_{10}+			
WakeC[a]	64	26	0.00	.998	0.000
CAR	56	19	2.09	.153	0.029
BedC[b, c]	95	35	3.20	.076^{+}	0.025
SlopeC[a,c]	64	26	2.16	.145	0.026
DayC[b,d]	96	36	0.03	.856	0.000
HCC	76	19	3.93	.050*	0.042
	EtG_{112}−	EtG_{112}+			
WakeC[a]	73	17	0.00	.994	0.000
CAR	65	10	5.30	.025*	0.070
BedC[b, c]	109	21	1.05	.308	0.008
SlopeC[a,c]	72	16	0.64	.427	0.008
DayC[b,d]	110	22	1.39	.240	0.011
HCC	85	10	3.61	.060^{+}	0.039

Note: All analyses were controlled for child age, maternal psychopathology, and birth weight as covariates, additionally following covariates were adjusted in single analyses: [a]antibiotics intake in the 6 months before sample collection, [b]time between first and last sample, [c]mean Apgar score, [d]school-day yes/no. EtG = Ethyl glucuronide.

Abbreviations: BedC, bedtime cortisol; CAR, cortisol awakening response; DayC, total cortisol release throughout the day; DayC, daytime cortisol; HCC, hair cortisol concentration; SlopeC, daily slope; WakeC, wakening cortisol.

$^{+}p < .10$,

$^{*}p < .05$.

TABLE 5 CAR-related sampling behavior of EtG_{112}− ($n = 65$) and EtG_{112}+ ($n = 10$) children

	EtG_{112}−	EtG_{112}+			
	M (*SD*)	*M* (*SD*)	*t*	*p*	*d*
Waking (time)[a]	7:32 (0:58)	8:02 (0:58)	−1.53	.130	0.36
Waking to *t*1 (min)	5.86 (5.12)	5.10 (3.25)	0.45	.653	0.11
*t*1 (time)	7:37 (0:59)	8:07 (0:58)	−1.48	.143	0.35
*t*2 (time)	8:08 (0:59)	8:38 (0:57)	−1.48	.144	0.35
*t*1 to *t*2 (min)	30.6 (3.36)	30.4 (2.59)	0.14	.890	0.00

Note: Sampling times: *t*1 = at awakening. *t*2 = 30 min after awakening. *t*3 = 12 a.m. *t*4 = 5 p.m. *t*5 at bedtime.

Abbreviations: CAR, cortisol awakening response; EtG, ethyl glucuronide.

[a]93% ($n = 70$) of children took the samples on a weekend or holiday day.

psychopathology. Additionally, there was no significant correlation of negative CARs with the wakening time ($r = .045$, $p = .612$) or the time from wakening to *t*1 ($r = -.097$, $p = .272$).

In the whole sample, cortisol levels were not associated with child psychopathology (Table 2). When considering only children with EtG concentrations ≥10 or ≥112 ng/g, and integrating confounders in partial correlations, child-relevant cortisol levels (ANCOVA $p < .10$) were associated with child psychopathology at medium effect size, but non-significant (Table 6). Specifically, higher cortisol levels at bedtime, a flatter CAR, and lower HCC levels were associated with more clinical symptoms, especially in the EtG_{112}+ children.

4 | DISCUSSION

The present study investigated the associations of prenatal alcohol consumption with preschool children's HPA axis activity in saliva (24-hr period) and hair (1 month) samples. Prenatal alcohol exposure was operationalized by the meconium biomarker EtG and additionally by maternal self-reports during the third trimester of pregnancy. Two EtG cut-offs (10 and 112 ng/g) were applied.

For both cut-offs (≥10 ng/g statistically significant and ≥112 ng/g significant by trend), exposed children showed a reduced HCC. The effect was small. For the 112 ng/g cut-off, the CAR was significantly decreased. Additionally, correlational data showed a dose-dependent effect for the CAR: the higher the meconium EtG level, the lower the cortisol level at *t*2 and consequently the CAR. These findings correspond to the results of Eichler et al. (2018) who found dose-response correlations for meconium EtG and child symptoms of attention-deficit hyperactivity disorder in preschool age. Particularly within the EtG_{112}+ positive group, the basal (CAR) and cumulative (HCC) cortisol parameters were associated with more clinical symptoms in the children at small/medium effect size. This biological association was not observed in the total sample or the control group. Therefore, the present study not only showed the associations of intrauterine alcohol exposure with HPA axis activity, but also examined the functional relevance of this developmental challenge for the affected children. We conclude that meconium EtG concentrations above our cut-offs are related to altered cumulative cortisol concentrations and that higher meconium EtG levels additionally alter children's daily cortisol profile. These findings support previous research (based on maternal self-report) (Pearson et al., 2015) that intrauterine alcohol exposure is a risk factor for an altered HPA axis development and provide the first evidence that meconium EtG can be used as an effective biomarker for such exposure. Additionally, our results support previous research findings that the EtG biomarker of intrauterine alcohol exposure can be used as a predictor of child development (Eichler et al., 2018; Frey et al., 2018).

In comparison to unexposed controls, the cortisol concentrations in the affected groups were consistently lower—especially the cumulative cortisol concentrations in hair. In earlier studies, the HPA axis dysregulation associated with prenatal alcohol consumption was mostly marked by hypercortisolism and greater activation of the HPA system in children. Earlier, authors, who tested basal cortisol

TABLE 6 Partial correlations r_p (p; df) of cortisol indices with child clinical symptoms in EtG+ groups

	n	Child total psychopathology	Child emotional problems	Child conduct problems
EtG ≥10 ng/g				
BedC[a,b]	35	.313[+] (.092; 28)	.036 (.850; 28)	.327[+] (.078; 28)
HCC	19	.035 (.879; 14)	−.204 (.449; 14)	−.083 (.761; 14)
EtG ≥112 ng/g				
CAR	10	−.401 (.372; 5)	−.642 (.120; 5)	−.257 (.578; 5)
HCC	10	−.471 (.286; 5)	−.660 (.107; 5)	−.467 (.291; 5)

Note: Total child psychopathology/emotional and conduct problems: Strength and Difficulties Questionnaire (Goodman, 2001). All correlations were controlled for child age, maternal psychopathology, and birth weight as covariates, additionally following covariates were added for bedtime cortisol: [a]time between first and last sample, [b]mean Apgar score.

Abbreviations: BedC, bedtime cortisol; CAR, cortisol awakening response; EtG, ethyl glucuronide; HCC, hair cortisol concentration.

[+]$p < .10$.

levels in older children, studied age heterogeneous groups (Keiver et al., 2015; McLachlan et al., 2016). The inclusion of a wide range of ages can be considered a confounder since cortisol activity is highly age-dependent (Kiess et al., 1995). For example, a meta-analysis in 2008, integrating 72 studies on childhood and adolescence basal cortisol activity, found preschoolers with externalizing symptoms to display a hypercortisolism while elementary schoolers had a hypocortisolism. The authors emphasize that HPA axis undergoes changes during development (Alink et al., 2008). In a prospective cohort study, Karlen and colleagues showed that child cortisol levels in hair continuously decreased from 1 to 8 years of age (Karlen, Frostell, Theodorsson, Faresjo, & Ludvigsson, 2013). It is widely accepted that the HPA axis is altered after prenatal alcohol exposure; however, the interindividual and age-dependent biological variance seem to be quite large during childhood. This variance is reflected in a meta-analysis, combining the outcomes of 19 studies on fetal programing and cortisol secretion in childhood, which noted no differences for the relation between programing variables and the direction of cortisol secretion (hyper- or hypocortisolism) in terms of effect size (Pearson et al., 2015).

Despite variation in the literature, there are studies reporting the phenomenon of basal hypocortisolism in children with emotional symptoms: saliva specimens (Chen, Raine, Soyfer, & Granger, 2015; Ruttle et al., 2011) and hair specimens (Lu, Pan, Ren, Xiao, & Tao, 2018). For emotional disorders in adulthood, there are also studies which describe a hypocortisolism in both saliva and hair specimens (Pochigaeva et al., 2017; Steudte et al., 2011).

The most significant difference we found was a reduced CAR in alcohol exposed children. We tested potential confounders carefully, for example, later awakening time for the exposed children, but found no evidence for methodological explanations. Thus, we interpret the CAR differences between exposed versus non-exposed children as a consequence of the intrauterine alcohol exposure. In our sample, the reduced CAR was associated with more emotional symptoms within the EtG_{112}+ group, which corresponds with earlier studies describing hypocortisolism in children with emotional symptoms (Chen et al., 2015; Lu et al., 2018; Ruttle et al., 2011). In our data, there was no statistical significance for this correlation, but a practical relevance, primarily due to the small sample size: there were only 10 EtG_{112}+ children with valid CAR values. Therefore, the results have to be interpreted with caution.

We found reduced HCCs in EtG+ children in comparison to the controls. Within the EtG_{10}+ (small effect) and the EtG_{112}+ (medium effect) groups, the HCC levels were negatively correlated with child emotional symptom ratings. These correlations were again non-significant, however, of practical relevance. We interpret that an altered HPA axis activity is a risk factor for child development. Nevertheless, according to the diathesis stress model (Zuckerman, 1999), this vulnerability only results in clinical symptoms when the child was prenatally exposed to alcohol—constituting an additional risk factor. An actual review of Koss and Gunnar emphasizes the role of HPA axis as a stress-mediating mechanism between various (not only prenatal) forms of childhood adversity and psychopathology (Koss & Gunnar, 2018).

Currently, there are no studies linking meconium EtG levels with prenatal drinking levels. Thus, the amount of consumed alcohol leading to altered cortisol levels in children remains undefined. However, there are results for other meconium ethanol metabolites from high-risk samples where prenatal alcohol consumption is not as stigmatized. Bearer et al. (Bearer et al., 2003) described that mothers of fatty acid ethyl esters (FAEE)-positive children drank an average of 10 drinks/week and not fewer than 4 drinks/week or a minimum of 1.5 ounces absolute alcohol per drinking occasion (equal to 1 L beer or 0.5 L wine). Derauf, Katz, & Easa, (2003) reported that one or two drinks per day were not enough to find positive FAEE meconium concentrations. Designing studies, which can link EtG meconium levels with maternal prenatal drinking amounts, is challenging because of maternal self-report bias. Self-reporting studies are likely most relevant for cultures or cohorts where alcohol drinking during pregnancy is not as stigmatized.

As already mentioned in the introduction, the use of meconium EtG levels is only one of the many methods available to quantify

intrauterine alcohol exposure in a newborn's biological matrix. Additionally, the examination of HPA axis activity is one of the many methods to determine developmental consequences after intrauterine alcohol exposure. Future research has to clarify many of these pathways to fully understand the underlying mechanisms and develop the proper early diagnostics for affected children. It cannot be ruled out if there are other factors besides prenatal alcohol exposure which are associated with EtG levels and which are responsible for differences in HPA axis activity; however, we tried to reduce alternative explanations by taking relevant confounders into account.

5 | CONCLUSION

We found associations of intrauterine alcohol exposure with preschool child HPA axis dysregulation during 1 day and during 1 month. These associations were partly dose-dependent. The alterations were of functional relevance: affected children had higher clinical symptoms when the HPA axis was dysregulated. This study underlines the importance of offering treatment to women who consume alcohol during pregnancy and/or early support for affected children. The results should motivate future research to further validate EtG as a biomarker of intrauterine alcohol exposure—in order to someday be used in obstetric practice to evaluate a newborn's alcohol-related impairments.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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