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Fetal Alcohol Syndrome: Changes in Craniofacial Form With Age, Cognition, and Timing of Ethanol Exposure in the Macaque

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ABSTRACT One component of the fetal alcohol syndrome (FAS) facial phenotype is a frontonasal anomaly characterized by a thin upper lip and a smooth philtrum. The expression of this anomaly can diminish with age and occurs infrequently in prenatal alcohol-exposed individuals. This study sought to explain these observations. Standardized craniofacial cephalograms of 18 nonhuman primates exposed weekly to ethanol or sucrose solution in utero were measured at ages 1, 6, 12, and 24 months to assess skeletal changes in craniofacial form with age, cognition, and timing of ethanol exposure. The data suggest that there may be a critical period for induction of alcohol-induced craniofacial alterations that occurs very early in gestation and is very short in duration (gestational days 19 or 20). The alterations were scarcely detectable at age 1 month, were most prominent at 6 months, and diminished progressively at 12 and 24 months in the macaque. The appearance and disappearance of the thin upper lip and smooth philtrum may be explained by underlying changes in skeletal structure with age. The infrequent occurrence of the FAS frontonasal anomaly may be explained, in part, by its short critical period of induction. *Teratology 59:163–172, 1999.* © 1999 Wiley-Liss, Inc.

Fetal alcohol syndrome (FAS) is a permanent birth defect caused by maternal consumption of alcohol during pregnancy (Jones et al., '73, '74; Clarren and Smith, '78). Central nervous system dysfunction, growth deficiency, and a unique cluster of minor facial anomalies characterize FAS. Although FAS is a lifelong disability, the physical features are not always expressed throughout life.

The FAS facial phenotype is typified by small palpebral fissures and a complex lower frontonasal anomaly described by a thin upper lip and a smooth philtrum (Jones et al., '73; Astley and Clarren, '96). The philtrum is the area between the vermilion border of upper lip and subnasion. These frontonasal features are often, but not always, minimally expressed at birth, maximally expressed in childhood, and diminish again in adulthood. Although this variable presentation with age is documented anecdotally in the literature (Spohr et al., '87, '93; Streissguth et al., '91), there are no estimates as to how often these features change with age and there is little understanding of the morphological basis for this variation. The lip and philtrum are soft-tissue structures. One could speculate that the soft-tissue changes are secondary to changes in the underlying bony structures. Much like a smile can stretch a deeply grooved philtrum and thick upper lip into a smooth philtrum and thin upper lip (Fig. 1), so might underlying pressure from early overgrowth of the premaxilla.

Although the facial features are a key diagnostic component of the syndrome, they are minor anomalies, which are usually of little medical consequence to the individual. Of greater significance is the fact that the facial anomalies are midline anomalies derived from the anterior frontal neural crest primordia of the early forebrain. It has long been speculated that some midline facial anomalies are pathognomonic of brain malformation (i.e., the face predicts the brain) (DeMeyer, '75). This speculation is supported by the presence of a proportional increase in midventral forebrain deficiencies and the severity of facial dysmorphia in mice exposed to ethanol early in gestation (Sulik, '84). Earlier work by Sulik and Johnston ('82) also provided compelling evidence that the critical period for the induction of FAS-like craniofacial malformations occurs very early in gestation (gestational day 7 in the mouse, the primitive streak stage in embryogenesis) and is very short in duration (no more than a few hours in the

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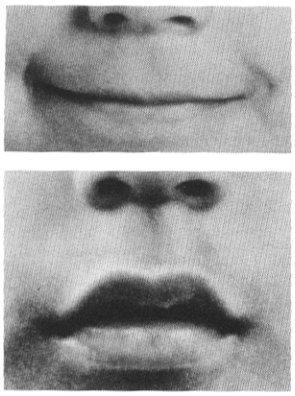

Fig. 1. Same individual with (**top**) and without (**bottom**) a smile, demonstrating how a smile can transform a deeply grooved philtrum (Likert rank = 2) (Astley and Clarren, '96) and full upper lip (Likert rank = 1, lip circularity [perimeter^2/area] = 40.9) (Astley and Clarren, '96) into a smooth philtrum (Likert rank = 4) and thin upper lip (Likert rank 5, lip circularity [perimeter^2/area] = 191.0).

mouse). Sulik and Johnston ('82) speculated that severe cases of FAS represent the mild end of the holoprosencephaly spectrum. Indeed, the only known nonhuman primate with holoprosencephaly was born after alcohol exposure in this project (Siebert et al., '91).

As form follows function, one could speculate that midline facial anomalies might also serve as markers for cognitive dysfunction. Although there is evidence to suggest that the magnitude of expression of the FAS facial phenotype is correlated with the severity of cognitive dysfunction (Jones et al., '73; Streissguth et al., '78; Streissguth and Dehaene, '93; Majewski, '81), these observations may be biased because the diagnostic criteria for FAS require the presence of both facial features and central nervous system (CNS) dysfunction.

An opportunity to assess variation in craniofacial form with timing of prenatal ethanol exposure, age, and cognitive dysfunction became available with the collection of standardized serial cephalometric radiographs of nonhuman primates (*Macaca nemestrina*) exposed to ethanol during gestation in a previous study (Clarren and Astley, '92; Clarren et al., '92). The primate model provides accurate documentation of timing and level of ethanol exposure, a controlled postnatal rearing environment, and a comprehensive battery of cognitive assessments, conditions which cannot be replicated in a human population (Clarren et al., '87, '88, '92; Clarren and Astley, '92; Astley et al., '95; Sirianni and Swindler, '85).

The present investigation was undertaken to address the following questions: 1) Does weekly prenatal ethanol exposure (1.8 g/kg maternal weight) result in detectable craniofacial malformations in *Macaca nemestrina* offspring? If malformations are detectable: 2) Is the primitive streak stage of embryogenesis a critical period of induction? 3) Does the magnitude of expression of alcohol-related craniofacial malformations change with age? 4) Is cognitive impairment correlated with the magnitude of craniofacial malformation?

MATERIALS AND METHODS

Subjects

A standardized series of cephalometric radiographs were collected at age 1, 6, 12, and 24 months on 18 nonhuman primates (*Macaca nemestrina*) who had been exposed to ethanol or a control solution of sucrose weekly during gestation (Clarren and Astley, '92) (Table 1).

Ethanol exposure

Details of timed-mating procedures, dosing schedules, and management of pregnancies were presented previously (Clarren and Astley, '92). Briefly, the animals were distributed across four exposure groups: 1) offspring exposed weekly to ethanol in the first 3 weeks of gestation, 2) offspring exposed weekly to ethanol in the first 6 weeks of gestation, 3) offspring exposed weekly to ethanol throughout the 24 weeks of gestation, and 4) a control group exposed weekly to sucrose solution, isocaloric and isovolemic to the ethanol dose, throughout gestation. To control for handling, dams in the 3- and 6-week ethanol groups received the sucrose solution weekly in all subsequent weeks of gestation.

Solutions were delivered to the dams via soft nasogastric tubes. All ethanol-exposed dams received ethanol at 1.8 g/kg maternal body weight, which resulted in mean peak plasma ethanol concentrations of 223 $\pm$ 28 mg/dl. These weekly dosing schedules were established to mimic the most common pattern of female drinking, i.e., weekend social drinking which often stops upon confirmation of pregnancy.

Cephalograms

Standardized lateral and frontal cephalograms of each offspring were obtained with the sedated animal seated in a cephalostat designed for nonhuman primate cephalometry (Sirianni and Swindler, '85). Kodak X-Omat TL industrial, high-resolution film was used. The X-ray beam was centered along the Frankfort horizontal plane for both the lateral and frontal cephalograms with a tube-film distance of 49 cm and a subject-film distance of 14 cm. Cephalograms were taken at age 1, 6, 12, and 24 months. This age distribution is approximately equivalent to age 4 months, 2 years, 4–6 years, and 8–10 years in the human.

Each frontal and lateral cephalogram was captured at 640 $\times$ 480 pixel resolution on a 256-unit gray scale using Optimas image acquisition and enhancement computer software (Optimas Corp., Edmonds, WA). The software was used to mark and derive the X, Y Cartesian coordinates of 19 standardized skeletal landmarks (Fig. 2). Basion could not be reliably identified in the cephalograms at age 1 month. A cleared skull with lead-marked landmarks was radiographed to serve as a guide for landmark identification. The X, Y coordinates

TABLE 1. Selected characteristics of study population (18 *Macaca nemestrina*)

Offspring ID no.		Ethanol-exposed on gestational days 19 or 20	Weeks of ethanol exposure*	Cognitive impairment score**	Gender	Postnatal age (months) at X-ray, postconceptional age (days)			
No.	Eartag					1	6	12	24
45	CF	Yes	3	8	M	212	351	539	910
49	RG	Yes	6	67	F	198	351	532	917
50	SA	Yes	6	33	M	203	351	532	909
56	SI	Yes	24	18	F	196	350	533	897
40	DX	No	0	0	F	199	354	535	900
34	LP	No	0	10	F	197	352	554	962
35	OH	No	0	10	F	199	352	543	899
36	SB	No	0	0	F	205	353	539	914
38	TU	No	0	0	F	200	354	539	902
39	VO	No	0	8	F	200	354	536	906
41	NT	No	3	20	M	198	354	537	902
43	XF	No	3	8	F	201	354	548	914
44	AE	No	3	8	M	204	351	534	918
46	ER	No	3	33	F	197	363	534	904
48	KX	No	6	20	F	200	354	537	984
51	SC	No	6	8	M	197	352	538	906
52	SR	No	6	25	F	199	352	534	911
55	SH	No	24	33	M	201	355	538	902

*Weekly ethanol exposure for first 3, 6, or 24 weeks of gestation. Maternal dose, 1.8 g/kg ethanol.
**Represents percent of cognitive and behavioral tests failed (Clarren and Astley, '92).

of the landmarks were used to calculate 17 linear and 3 angular measurements (Table 2). These measures were selected to best capture the craniofacial form associated with FAS. Head circumference (OFC) and body weight were measured at the time of each X-ray. Brain weight and volume were recorded at the time of sacrifice (age 4.0–5.3 years).

Cognitive assessments

Motor, cognitive, behavioral, and physical development was documented from birth through age 14 months. The comprehensive assessment battery was developed and has been administered at the Infant Primate Research Laboratory for over 20 years. The content and timing of the assessments are described in detail in previous reports (Clarren et al., '88, '92). An unweighted composite score reflecting each animal's developmental impairment was derived by dividing the number of assessments an animal failed by the number of assessments used to summarize each animal's development in Figure 1 as presented in Clarren et al. ('92). Failure for each assessment was defined as performance >2 SDs below the mean performance of the control animals.

Analysis

Repeated-measures analysis of covariance, with adjustment for gender, was used to compare mean distance and angular craniofacial measures between the ethanol-exposed and unexposed groups at age 1, 6, 12, and 24 months. To evaluate the impact of timing of exposure on outcome, the analysis was repeated three times with the animals' exposure status classified in three different ways: 1) in their original four groups based on *duration* of exposure (0, 3, 6, or 24 weeks), 2) in two groups based on *exposed / not exposed* (the 3-, 6-, and 24-week groups were combined and compared to the control group), and 3) in two groups based on *exposed or not exposed on gestational days 19 or 20.* The rationale for these three different exposure classifications is as follows. Facial development is essentially complete by the seventh week of gestation in the macaque, with a potential critical period of vulnerability occurring as early as week 3 (Sulik and Johnston, '83). Since all ethanol-exposed animals started their weekly exposures within the first 13 days of gestation, we hypothesized that classifying the animals by duration of exposure would be least sensitive for identifying craniofacial contrasts, classifying them based on exposed/not exposed would be more sensitive, and that classifying them based on who received ethanol exposure specifically on gestational days 19 or 20 would be the most sensitive. Gestational days 19 and 20 in the macaque correspond with the critical period of induction for FAS facial malformations (gestational day 7 or the primitive streak stage) observed in the mouse (Sulik and Johnston, '83).

Multiple linear regression analyses with adjustment for gender were conducted to detect correlations between craniofacial measures and the overall cognitive impairment score. These analyses were repeated at each of the four ages (1, 6, 12, and 24 months).

It is important to note that the original primate study from which these data were derived was not designed to address the specific questions presented in this study. Due to the small number of animals in each exposure group, only large contrasts between groups were statistically detectable. In general, when a study's power to detect a clinically meaningful contrast drops below 80%, the study is at risk for falsely declaring an absence

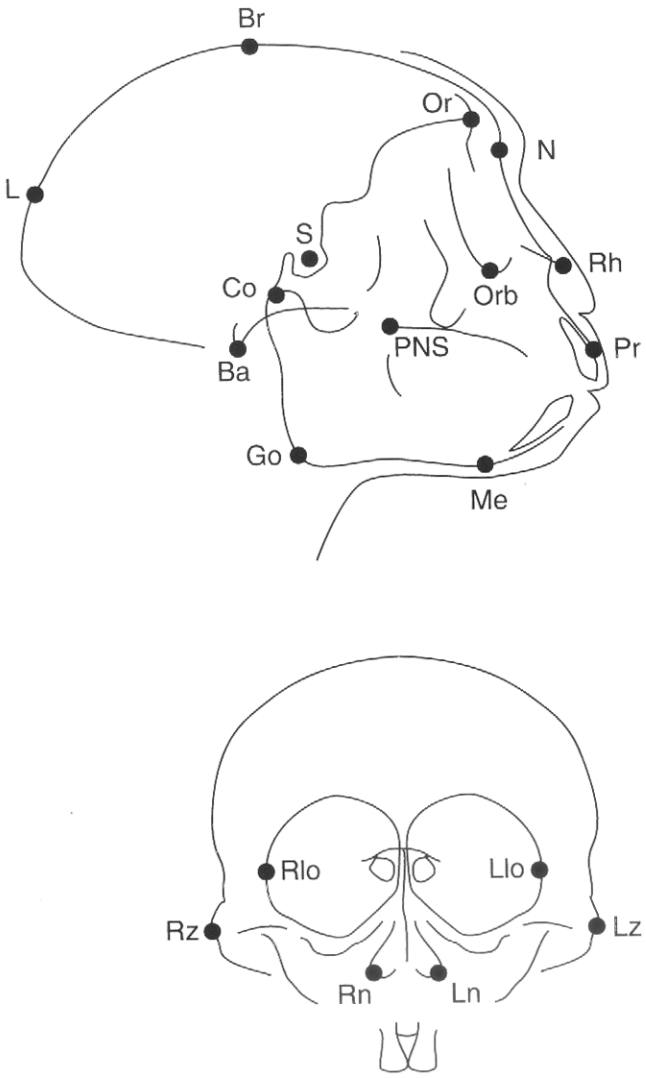

Fig. 2. Landmarks on lateral radiographs. L, lambda, most superior point on the lambdoid suture; Br, bregma, point at junction of coronal and sagittal sutures; S, sella, center of pituitary fossa of the sphenoid bone; Go, gonion, midpoint of angle of mandible; PNS, posterior nasal spine, most posterior point at the sagittal plane on the bony hard palate; Co, condylion, most posterior, superior point on curvature on average of right and left outlines of condylar heads; Ba, basion, most inferior, posterior point on anterior margin of foramen magnum; Or, junction of orbital roof and inner table of the frontal bone; Orb, orbitale, lowest point on average of right and left borders on the bony orbit; Me, menton, most inferior point on the symphyseal outline; N, nasion, junction of frontonasal suture at the deepest point on curve at bridge of nose; Rh, rhinion, most superior, inferior point of nasal bone; Pr, prosthion, most anteroinferior point on upper alveolar margin. Landmarks on frontal radiographs. Rlo, right lateral orbit, at point where zygomatic and frontal bones meet; Llo, left lateral orbit, at point where zygomatic and frontal bones meet; Rz, right zygoma, most lateral portion of zygomatic arch; Lz, left zygoma, most lateral portion of zygomatic arch; Rn, right nasal cavity, most lateral portion of nasal cavity; Ln, left nasal cavity, most lateral portion of nasal cavity.

of contrast between groups. When between-group contrasts observed in this study failed to achieve statistical significance, these outcomes were accompanied by estimates of the minimum effect sizes that could have been detected at 80% power (Bornstein et al., '97).

TABLE 2. Morphometric measurements*

Lateral cephalometric films	
Cranial measurements	
Cranial height	Ba-Br
Anterior cranial base	S-Or
Posterior cranial base	S-Ba
Posterior cranium	S-L
Cranial vault height	S-Br
Cranial length	L-Or
Cranial base angle	Ba-S-Or
Sagittal cranial area (at midline)	L-S-Or-Br
Midface measurements	
Midface height	N-Pr
Posterior midface height	S-PNS
Facial depth I	S-Rh
Facial depth II	S-Orb
Facial depth III	S-Pr
Palatal length	PNS-Pr
Facial angle	Or-Pr-PNS
Anterior cranial base to facial plane	S-Or-Pr
Mandibular measurements	
Mandibular body length	Go-Me
Mandibular ramus height	Go-Co
Frontal cephalometric films	
Interorbital width	Rlo-Llo
Bizygomatic width	Rz-Lz
Nasal cavity width	Rn-Ln

*Abbreviations used are spelled out in Figure 2.

Measurement precision

Landmark identification was repeated, 2 weeks apart, on 10 randomly selected cephalograms. The measurements for each set of radiographs were compared and revealed a high degree of precision. The method error was determined by the equation of Dahlberg ('40), where the sum of the squared differences is divided by two times the number of measurements and the square root is calculated. The method error was 0.27 mm for linear and 1° for angular measurements. Error calculated as a percentage of the measurements examined was less than 2%.

RESULTS

Effect of ethanol exposure and age on craniofacial form

When the cephalometric measurements were compared between the 0-week (n = 6), 3-week (n = 5), 6-week (n = 5), and 24-week (n = 2) ethanol exposure groups to test whether craniofacial form and brain size varied as the duration of exposure increased, only one statistically significant trend was detected. At age 6 months, mean cranial length (L-Or) increased linearly from 71.4 mm to 73.9 mm to 75.1 mm to 76.7 mm as duration of exposure increased from 0 to 3 to 6 to 24 weeks, respectively ($P = 0.03$). It is worth noting that the same pattern and relative magnitude of change in cranial length were also observed at ages 1, 12, and 24 months. Increased variability in cranial length dropped the power of detection to less than 35%, which may explain why these trends failed to achieve statistical significance. Mean head circumference was comparable (maximum contrast 5%) between all four groups across

all ages. Mean brain weight and volume were comparable (maximum contrast 10%) between all four groups at sacrifice. This series of analyses had 80% power to detect a ≥2 mm (or 5%) and a ≥2° (or 10%) incremental change in linear and angular craniofacial measures, a ≥9 mm (or 4%) incremental change in OFC, and an ≥8 g or cc (or 10%) incremental change in brain weight or volume.

When the analyses were repeated with the animals reclassified as exposed (n = 12) and not exposed (n = 6) to ethanol, again, only a few contrasts in craniofacial form were detected. All linear measures at each of the four ages were consistently greater in the ethanol-exposed animals relative to the unexposed animals. On average, the 20 linear measures were 3.5 ± 4.9% greater at age 1 month, 4.5 ± 3.1% greater at age 6 months, 2.2 ± 2.3% greater at age 12 months, and 2.5 ± 2.6% greater at age 24 months in the ethanol-exposed animals relative to the nonexposed animals. There was no consistent pattern or direction of change in the three angular measures across the four ages. The only contrasts which achieved statistical significance included an increased orbital distance (Rlo-Llo) by 1.7 mm (or 4%) at ages 6 and 24 months ($P = 0.01$), and an increased cranial length (L-Or) by 3.5 mm (or 5%) at age 6 months ($P = 0.04$) in the ethanol exposed animals relative to the unexposed animals. Mean head circumference was comparable (2–4% difference) between the exposed and unexposed groups across all ages. Mean brain weight and volume were comparable (3% difference) across the two groups at sacrifice. These analyses had an estimated 80% power to detect a ≥3 mm or degree (or 5–10%) difference between the craniofacial measures, a ≥15 mm (or 6%) difference in OFC, and a ≥15 g or cc (or 20%) difference in brain weight or volume between the two exposure groups.

When the analyses were repeated one more time with the animals reclassified as exposed (n = 4) or not exposed (n = 14) to ethanol on gestational days 19 or 20 (the period comparable to gestational day 7 in the mouse found to be important by Sulik and Johnston, '82), substantially more contrasts were identified (Table 3, Fig. 3). On average, the 20 linear measures were 2.8 ± 3.8% greater at age 1 month, 4.8 ± 5.1% greater at age 6 months, 3.2 ± 3.7% greater at age 12 months, and 5.4 ± 1.9% greater at age 24 months in the animals exposed on days 19 or 20 relative to those not exposed on days 19 or 20. On average, the three angular measures were 0.1 ± 2.6% smaller at age 1 month, 4.6 ± 3.0% smaller at age 6 months, 1.1 ± 1.3% smaller at age 12 months, and 0.3 ± 1.4% smaller at age 24 months in the animals exposed on days 19 or 20 relative to those not exposed on days 19 or 20. The number of statistically significant contrasts in craniofacial form between the two groups of animals was minimal at age 1 month, increased substantially at age 6 months, and diminished again at age 12 and 24 months. More specifically, at age 1 month, only 3 of the 20 craniofacial measures were significantly different in the animals exposed on days 19 or 20: head length (L-Or) was 3.7 mm (or 5.5%) greater, OFC was 8.0 mm (or 3.7%) greater, and facial depth (S-Pr) was 2 mm (or 5.7%) greater. At age 6 months, 6 of the 20 measures were significantly different in the animals exposed on days 19 or 20. These included head length (L-Or) greater by 4.2 mm (or 5.8%), midface height (N-Pr) greater by 4.5 mm (or 17.6%), two measures of facial depth (S-Rh greater by 4 mm (or 11%), and S-Pr greater by 2.7 mm (or 6.3%), facial angle (Or-Pr-PNS) smaller by 4.7° (or 7.9%), and internasal width (Rn-Ln) greater by 1.5 mm (or 14.9%). At age 12 months, 3 of 20 measures were significantly greater in the animals exposed on days 19 or 20: cranial length (L-Or) by 4.4 mm (or 5.9%), posterior midface height (S-PNS) by 1.8 mm (or 10.6%), and internasal width (Rn-Ln) by 1.1 mm (or 10.3%). At age 24 months, 4 of the 20 measures were significantly different between the animals exposed and not exposed to ethanol on gestational days 19 or 20. These included cranial length (L-Or) greater by 5.8 mm (or 7.8%), OFC greater by 15.3 mm (or 5.9%), interorbital width (Rlo-Llo) greater by 1.4 mm (or 2.8%), and bizygomatic width (Rz-Lz) greater by 3.1 mm (or 7.3%). The mean brain weight in grams was 94.9 ± 6.3 vs. 84.8 ± 8.8 ($f = 3.2$, $P = 0.11$) in the animals exposed and not exposed to ethanol on gestational days 19 or 20, respectively. The mean brain volume in cubic centimeters was 88.0 ± 5.3 vs. 80.1 ± 8.1 ($f = 2.3$, $P = 0.16$) in the animals exposed and not exposed to ethanol on gestational days 19 or 20, respectively. This series of analyses had 80% power to detect a ≥3 mm (or 5%) or ≥3 degree (or 10%) difference between craniofacial measures, a ≥15 mm (or 5%) difference in OFC, and a ≥15 g or cc (or 20%) difference in brain weight or volume between the two exposure groups.

As further evidence that the critical period of induction may be gestational days 19 or 20 in the macaque, two additional exploratory analyses were conducted, comparing all craniofacial measures between animals exposed and not exposed to ethanol just prior to the critical period (gestational days 17 or 18) and comparing animals exposed and not exposed to ethanol just after the critical period (gestational days 21 or 22). No statistically significant contrasts were identified in either analysis.

Correlations between craniofacial form and cognition

The cognitive impairment score increased as craniofacial linear measures increased and craniofacial angular measures decreased (Table 4, Fig. 4). The directions of these correlations were consistent with the alcohol-related changes observed in the animals exposed to ethanol on gestational days 19 or 20. The proportion of craniofacial measures that correlated significantly with cognitive impairment increased with age: 12% at age 1 month, 21% at age 6 months, 37% at age 12 months, and 32% at age 24 months. The magnitude of the correlations also increased with age. At age 12 months, when the greatest number of correlations was observed, cognitive impairment increased significantly with in-

TABLE 3. Craniofacial contrasts between 4 animals that did and 14 animals that did not receive ethanol exposure on gestational days 19 or 20 (primitive streak stage or Carnegie stage 9)*

Craniofacial measure	Group	Postnatal age: 1 month Mean	(SD)	P	6 months Mean	(SD)	P	12 months Mean	(SD)	P	24 months Mean	(SD)	P
Cranial height (mm) Ba-Br	Control	Insufficient data			46.1	(1.4)	0.84	48.0	(1.7)	0.96	49.5	(2.3)	0.17
	Ethanol				46.4	(1.5)		48.0	(1.2)		51.5	(1.2)	
Anterior cranial base (mm) S-OR	Control	27.8	(1.0)	0.12	32.4	(1.7)	0.33	34.0	(1.4)	0.18	35.2	(1.7)	0.07
	Ethanol	28.9	(1.0)		33.6	(1.1)		35.3	(1.1)		37.1	(1.0)	
Posterior cranial base (mm) S-Ba	Control	Insufficient data			15.8	(1.1)	0.93	16.9	(0.9)	0.74	17.8	(1.1)	0.12
	Ethanol				15.9	(0.9)		17.0	(0.8)		18.8	(0.7)	
Posterior cranium (mm) S-L	Control	44.6	(1.6)	0.31	46.7	(1.7)	0.15	47.0	(1.7)	0.08	47.7	(2.0)	0.09
	Ethanol	45.7	(0.8)		48.2	(1.0)		48.7	(0.8)		49.8	(0.9)	
Cranial vault height (mm) S-Br	Control	32.8	(1.5)	0.60	35.8	(1.9)	0.63	36.5	(1.8)	0.89	37.9	(2.1)	0.56
	Ethanol	32.7	(2.1)		35.7	(2.0)		36.9	(1.4)		38.8	(2.3)	
Cranial length (mm) L-Or	Control	67.7	(2.1)	0.009	72.8	(2.6)	0.02	73.6	(2.0)	0.003	74.2	(2.8)	0.004
	Ethanol	71.4	(1.2)		77.0	(1.9)		78.0	(2.4)		80.0	(1.9)	
Cranial base angle (°) Ba-S-Or	Control	Insufficient data			166.5	(5.6)	0.05	166.8	(5.6)	0.41	170.6	(4.5)	0.43
	Ethanol				159.4	(7.1)		163.9	(4.5)		168.1	(4.9)	
Anterior cranial base to facial plane (°) S-Or-Pr	Control	79.3	(1.8)	0.09	80.6	(2.7)	0.21	83.1	(2.7)	0.86	84.3	(2.4)	0.50
	Ethanol	81.7	(3.4)		78.8	(5.0)		83.4	(2.9)		85.4	(3.2)	
Head circumference (mm)	Control	215.8	(6.3)	0.04	237.1	(7.9)	0.20	249.6	(9.2)	0.26	259.7	(8.8)	0.02
	Ethanol	223.8	(4.8)		243.8	(6.9)		256.5	(10.1)		275.0	(11.2)	
Midface height (mm) N-Pr	Control	22.1	(1.5)	0.74	25.5	(1.9)	0.005	28.3	(2.2)	0.44	36.1	(3.3)	0.36
	Ethanol	22.6	(0.5)		30.0	(2.9)		29.5	(5.4)		38.5	(5.9)	
Posterior midface height (mm) S-PNS	Control	11.4	(0.7)	0.07	15.9	(1.4)	0.21	17.0	(1.0)	0.04	19.5	(1.3)	0.10
	Ethanol	12.5	(1.4)		17.1	(1.8)		18.8	(2.1)		21.1	(2.1)	
Facial depth I (mm) S-Rh	Control	31.4	(1.3)	0.06	36.5	(1.5)	0.001	39.9	(1.3)	0.08	44.8	(2.0)	0.07
	Ethanol	33.2	(2.0)		40.5	(1.6)		42.2	(3.7)		47.8	(3.9)	
Facial depth II (mm) S-Orb	Control	23.1	(1.8)	0.11	28.5	(1.2)	0.72	30.0	(0.9)	0.12	32.3	(2.0)	0.34
	Ethanol	25.0	(1.4)		28.8	(0.5)		31.0	(1.1)		33.6	(0.9)	
Facial depth III (mm) S-Pr	Control	35.0	(1.4)	0.046	42.8	(1.8)	0.02	47.8	(1.4)	0.09	55.2	(2.9)	0.14
	Ethanol	37.0	(1.9)		45.5	(1.3)		50.0	(3.2)		58.1	(3.4)	
Palatal length (mm) PNS-Pr	Control	24.9	(1.2)	0.47	28.5	(1.5)	0.30	32.6	(1.3)	0.99	37.3	(2.2)	0.36
	Ethanol	25.6	(0.9)		29.4	(1.4)		32.5	(2.2)		38.4	(1.2)	
Mandibular body length (mm) Go-Me	Control	22.1	(1.4)	0.55	26.4	(1.9)	0.59	29.5	(1.4)	0.05	34.8	(3.0)	0.18
	Ethanol	22.7	(0.6)		27.3	(0.6)		31.2	(0.6)		37.0	(0.8)	
Mandibular ramus height (mm) Go-Co	Control	Insufficient data			20.7	(1.8)	0.68	24.5	(1.7)	0.53	27.0	(2.7)	0.18
	Ethanol				21.2	(1.3)		23.2	(1.5)		28.3	(2.0)	
Facial angle (°) Or-Pr-PNS	Control	61.8	(2.1)	0.39	59.6	(2.7)	0.007	56.1	(3.0)	0.45	49.0	(2.4)	0.79
	Ethanol	60.6	(3.4)		54.9	(5.4)		54.9	(5.4)		48.7	(5.6)	
Interorbital width (mm) Rlo-Llo	Control	39.9	(1.5)	0.38	44.5	(1.3)	0.14	47.1	(0.8)	0.16	50.6	(1.2)	0.03
	Ethanol	40.6	(1.0)		45.3	(0.5)		47.8	(1.1)		52.0	(0.8)	
Bizygomatic width (mm) Rz-Lz	Control	49.0	(2.4)	0.33	59.1	(2.2)	0.19	63.6	(2.1)	0.13	69.7	(2.3)	0.03
	Ethanol	50.2	(1.9)		60.5	(1.3)		65.4	(1.8)		72.8	(2.0)	
Internasal width (mm) Rn-Ln	Control	8.8	(1.5)	0.43	10.1	(1.2)	0.04	10.7	(1.1)	0.049	12.2	(1.0)	0.10
	Ethanol	8.1	(2.0)		11.6	(1.2)		11.8	(0.7)		13.3	(1.2)	

*SD, Standard deviation; P, repeated measures analysis of variance two-tailed P value after adjustment for gender. Craniofacial measure abbreviations are spelled out in Fig 2. Statistically significant outcomes are underlined.

creasing cranial length (L-Or), increasing posterior midface height (S-PNS), increasing facial depth (S-Rh, S-Orb, S-Pr), and decreasing facial angle (Or-Pr-PNS).

DISCUSSION

This investigation of craniofacial morphology in *Macaca nemestrina* exposed to ethanol in utero revealed significant craniofacial alterations and substantiates previous reports of teratogenesis with weekly 1.8 g/kg gestational ethanol exposure in nonhuman primates (Clarren et al., '88, '92). To achieve the maternal peak plasma ethanol levels recorded for this sample of nonhuman primates, the average woman would need to consume 6–9 beers in the course of a few hours. In brief summary, the results of this study suggest that there may be a critical period for induction of alcohol-induced craniofacial alterations that occurs very early in the macaque's gestation and is very short in duration (gestational days 19 or 20). The ethanol-induced craniofacial alterations were scarcely detectable at age 1 month, were most prominent at age 6 months, and diminished progressively at ages 12 and 24 months.

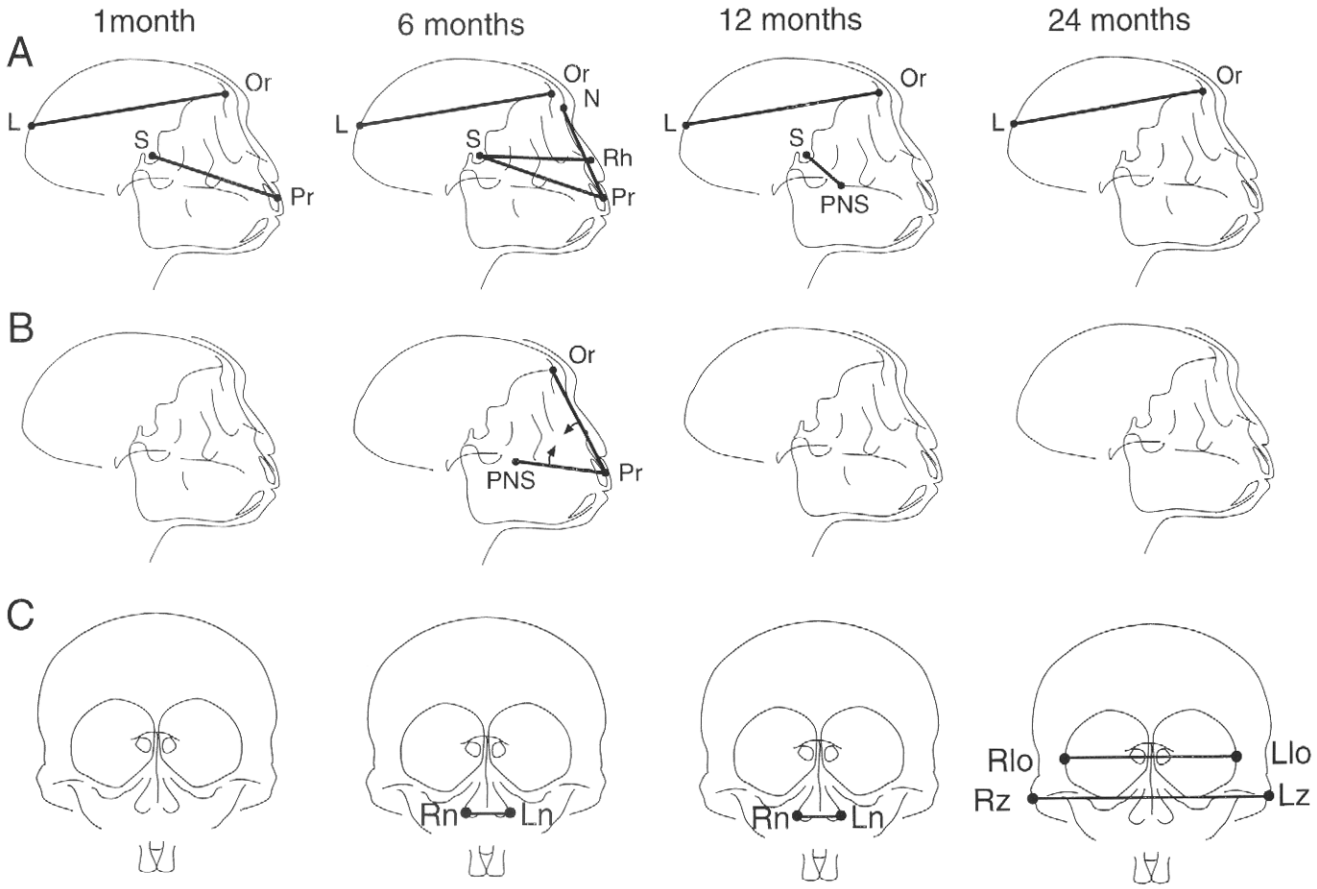

Fig. 3. Overview of craniofacial contrasts at age 1, 6, 12, and 24 months among 4 animals exposed to ethanol on gestational days 19 or 20 relative to 14 animals not exposed on gestational days 19 and 20. **A, C:** Straight lines reflect dimensions that were significantly greater at the $P < 0.05$ level in the 4 animals exposed on gestational days 19 or 20. **B:** The angular measure was significantly smaller in the 4 animals exposed on gestational days 19 or 20. Same abbreviations as in Figure 2.

Finally, the strong correlations observed between alcohol-induced craniofacial alterations and cognitive impairment suggest that midline facial anomalies may be sensitive indicators of brain dysfunction.

Timing of ethanol exposure

As we hypothesized, classifying the animals based on who did or did not receive ethanol exposure on gestational days 19 or 20 resulted in the maximum number of craniofacial contrasts, supporting Sulik ('84), who found that the critical period of induction for FAS-like facial anomalies occurred very early in gestation (gestational day 7 in the mouse, the primitive streak stage) and was very short in duration (just a few hours in the mouse). The findings from these two studies may explain, in part, why only an estimated 1–9% of women who are chronic alcoholics give birth to a child with FAS (Abel and Sokol, '87). A diagnosis of FAS requires the presence of the FAS facial phenotype. If the critical period of induction for the FAS facial phenotype is truly only 1 or 2 days long, even a frequent drinker could fail to expose her fetus on those days.

Sulik and Johnston ('82) also demonstrated that ethanol can induce holoprosencephaly in a mouse model, supporting the speculation that severe cases of FAS represent the mild end of holoprosencephaly. Interestingly, the only documented case of holoprosencephaly in a nonhuman primate occurred in this study, in an animal whose weekly ethanol exposure included exposure on gestational day 19 (Siebert et al., '91).

Craniofacial form and changes with age

The macaques exposed to ethanol on gestational days 19 or 20 had significantly longer midfaces (N-Pr) and protruded premaxillas (S-Rh, S-Pr, and S-PNS). These craniofacial alterations were barely detectable at age 1 month, were most strongly expressed at age 6 months, and progressively diminished at ages 12 and 24 months. These results are not only consistent with observations in the human population (Fig. 1), but may also explain why the soft-tissue anomalies associated with the FAS facial phenotype vary with age. It has long been our belief that the appearance and disappearance of the smooth philtrum and thin upper lip in individuals with

TABLE 4. Correlations between craniofacial measures over time with cognitive impairment score*

Craniofacial measure	Group	Postnatal age: 1 month Corr.	1 month R^2	1 month P	6 months Corr.	6 months R^2	6 months P	12 months Corr.	12 months R^2	12 months P	24 months Corr.	24 months R^2	24 months P
Cranial height (mm) Ba-Br	Control	Insufficient data			(−.25)	.60	0.35	(−.10)	0	0.71	(−.07)	.01	0.77
	Ethanol												
Anterior cranial base (mm) S-OR	Control	(−.15)	.02	0.54	(+.11)	.01	0.67	(+.10)	0	0.71	(+.09)	0	0.75
	Ethanol												
Posterior cranial base (mm) S-Ba	Control	Insufficient data			(−.29)	.08	0.28	(+.19)	.04	0.44	(+.19)	.03	0.46
	Ethanol												
Posterior cranium (mm) S-L	Control	(+.03)	0	0.96	(+.37)	.14	0.13	(+.36)	.13	0.14	(+.16)	.03	0.52
	Ethanol												
Cranial vault height (mm) S-Br	Control	(−.47)	.22	0.05	(−.34)	.11	0.18	(−.26)	.07	0.31	(−.25)	.07	0.31
	Ethanol												
Cranial length (mm) L-Or	Control	(+.31)		0.22	(+.54)	.30	0.02	(+63)	.40	0.005	(+.44)	.21	0.07
	Ethanol												
Cranial base angle (°) Ba-S-Or	Control	Insufficient data			(−.62)	.38	0.01	(−.36)	.13	0.15	(−.51)	.27	0.03
	Ethanol												
Anterior cranial base to facial plane (°) S-Or-Pr	Control	(+.58)	.34	0.01	(+.09)	0	0.73	(0)	0	0.99	(−.09)	0	0.99
	Ethanol												
Head circumference (mm)	Control	(+.07)	0	0.19	(+.19)	.03	0.46	(+.28)	.08	0.28	(+.41)	.17	0.10
	Ethanol												
Midface height (mm) N-Pr	Control	(−.15)	.02	0.56	(+.26)	.07	0.30	(+.48)	.23	0.04	(+.50)	.25	0.04
	Ethanol												
Posterior midface height (mm) S-PNS	Control	(+.37)	.14	0.13	(+.32)	.10	0.20	(+.46)	.21	0.057	(+.55)	.30	0.02
	Ethanol												
Facial depth I (mm) S-Rh	Control	(+.17)	.03	0.51	(+.43)	.18	0.08	(+.61)	.37	0.008	(+.58)	.33	0.01
	Ethanol												
Facial depth II (mm) S-Orb	Control	(+.19)	.04	0.47	(+.29)	.09	0.24	(+.53)	.28	0.03	(+.27)	.07	0.28
	Ethanol												
Facial depth III (mm) S-Pr	Control	(+.20)	.04	0.43	(+.46)	.21	0.055	(+.61)	.37	0.007	(+.45)	.20	0.06
	Ethanol												
Palatal length (mm) PNS-Pr	Control	(−.04)	.01	0.84	(+.22)	.05	0.39	(+.30)	.09	0.23	(+.17)	.03	0.50
	Ethanol												
Mandibular body length (mm) Go-Me	Control	(+.21)	.04	0.41	(+.32)	.10	0.20	(+.41)	.17	0.09	(+.24)	.06	0.34
	Ethanol												
Mandibular ramus (mm) Go-Co	Control	Insufficient data			(−.15)	.02	0.57	(−.11)	.10	0.65	(+.07)	0	0.79
	Ethanol												
Facial angle (°) Or-Pr-PNS	Control	(−.31)	.09	0.22	(−.34)	.12	0.16	(−.47)	.22	0.05	(−.48)	.23	0.04
	Ethanol												
Interorbital width (mm) Rlo-Llo	Control	(−.21)	.04	0.41	(+.21)	.03	0.45	(+.22)	.05	0.40	(+.36)	.08	0.14
	Ethanol												
Bizygomatic width (mm) Rz-Lz	Control	(−.25)	.06	0.31	(+.11)	.01	0.70	(+.28)	.08	0.27	(+.40)	.11	0.10
	Ethanol												
Internasal width (mm) Rn-Ln	Control	(−.42)	.18	0.08	(+.49)	.24	0.055	(+.45)	.20	0.07	(+.51)	.26	0.03
	Ethanol												

*Corr., Pearson correlation coefficient; R^2, proportion of variance in cognitive impairment score explained by craniofacial measure after adjustment for gender. Reported only when stepwise selection process in the multiple linear regression included craniofacial measure in the model. P, P-value for craniofacial measure partial correlation coefficient, if craniofacial measure was included in the model. Abbreviations for column 1 are spelled out in Fig 2. Statistically significant outcomes are underlined.

FAS may be secondary to alterations in underlying bony structure with age. Just as a smile can stretch a deeply grooved philtrum and full upper lip into a smooth philtrum and thin upper lip (Fig. 1), protrusion of the premaxilla could have a similar effect. The timing of the onset and regression of the premaxillary protrusion observed in this study closely corresponds with the timing of the appearance and disappearance of smooth philtrums and thin upper lips observed in the human population. It is interesting to note that Martin et al. ('96) identified distinct differences in the structure of the upper lip in a study comparing philtral development in the normal fetus with philtral development in specimens (including a specimen with prenatal alcohol exposure) lacking normal philtral landmarks. These findings, however, do not readily explain why philtrum smoothness varies with age. These contrasts in philtral muscular structure may explain the degree to which philtrums and upper lips are malleable by deformity due to underlying bone growth.

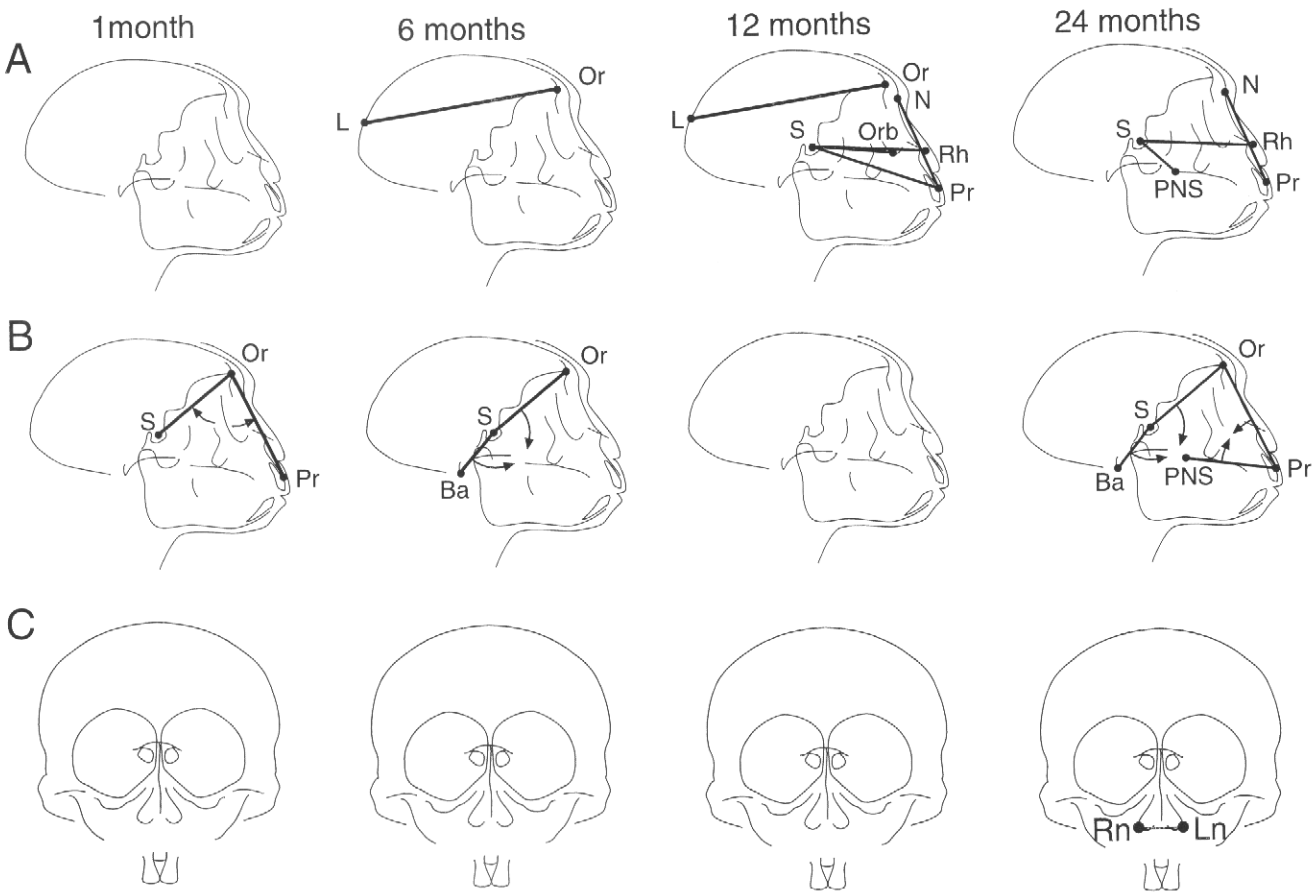

Fig. 4. Overview of craniofacial dimensions which were significantly correlated with cognitive impairment at age 1, 6, 12, and 24 months among all 18 animals ($P < 0.05$). **A, C:** All linear measures increased significantly as cognitive impairment increased. **B:** Angular measures changed in the direction indicated by arrows as cognitive impairment increased. Same abbreviations as in Figure 2.

Cranial size and shape

Interestingly, the OFCs in ethanol-exposed animals were consistently and often significantly larger than in the unexposed animals. This appears to be in contrast to the known relationship between in utero ethanol exposure and decreased brain size (Smith et al., '86; Autti-Ramo et al., '92). Clinically, OFC is used as a proxy measure for brain size, a proxy measure that is valid only if brain shape remains relatively constant. In this study, the increased OFC appears to be influenced more by increased cranial length (L-Or) than by increased brain size. Larger doses of ethanol initiated later in gestation have resulted in microcephaly and scaphocephaly in earlier nonhuman primate studies (Inouye et al., '85; Sheller et al., '88). Inouye et al. ('85) reported a tendency for increased cranial length in two macaques exposed to 2.5 g/kg and 4.1 g/kg ethanol on a weekly basis from 30 days postconception to birth. These findings suggest that ethanol may not only have an influence on the size of the cranium, but on cranial shape as well.

Correlations between midline craniofacial form and cognitive dysfunction

The strong correlations observed between the alcohol-induced craniofacial alterations and cognitive impairment further support the idea that midline craniofacial anomalies may be sensitive indicators of brain dysfunction (DeMeyer, '75). The FAS facial phenotype varies on a continuum and if measured on a continuous scale could serve as a more sensitive indicator of teratogenic outcome than the current practice of recording the FAS facial phenotype as simply present or absent. Preliminary analyses (unpublished) found strong, statistically significant correlations between increasing magnitude of expression of FAS facial phenotype and decreasing intelligence quotient (full-scale, performance, and verbal IQ) among children with prenatal alcohol exposure. The facial phenotype was measured on a continuous scale called the D-score (Astley and Clarren, '96) that reflects the combined magnitude of expression of palpebral fissure length, smooth philtrum, and upper lip thinness.

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