International Conference and Exhibition on
Molecular Medicine-2015
OMICS International
Molecular Medicine and Diagnostics
August 24-26, 2015 London, UK

Methylation alterations of LINE-1 & imprinting genes related to folate deficiency and risk of neural tube defects

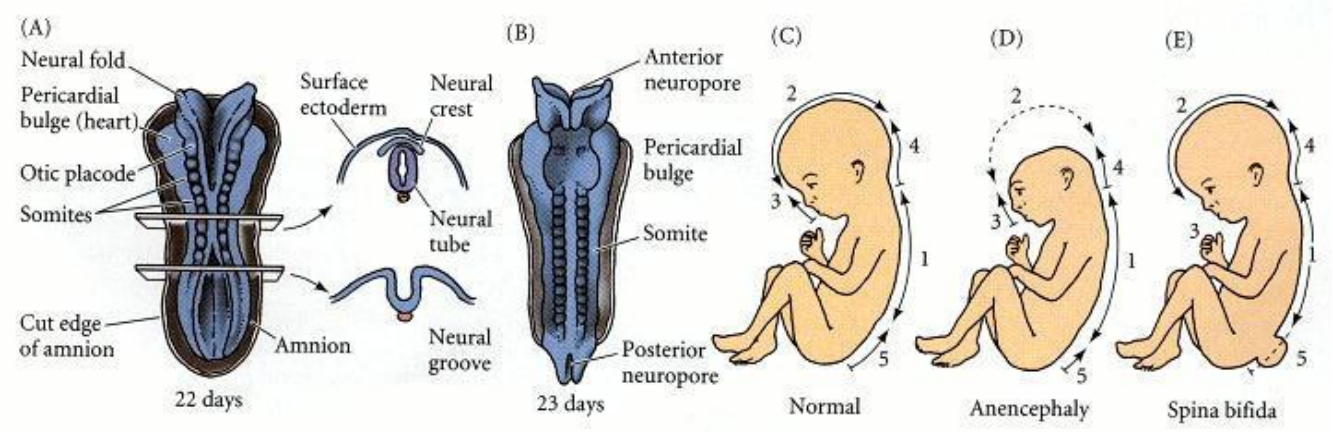
Wang Li PhD

Medical Genetic Department,
Capital Institute of Pediatrics, China



Neural Tube Defects

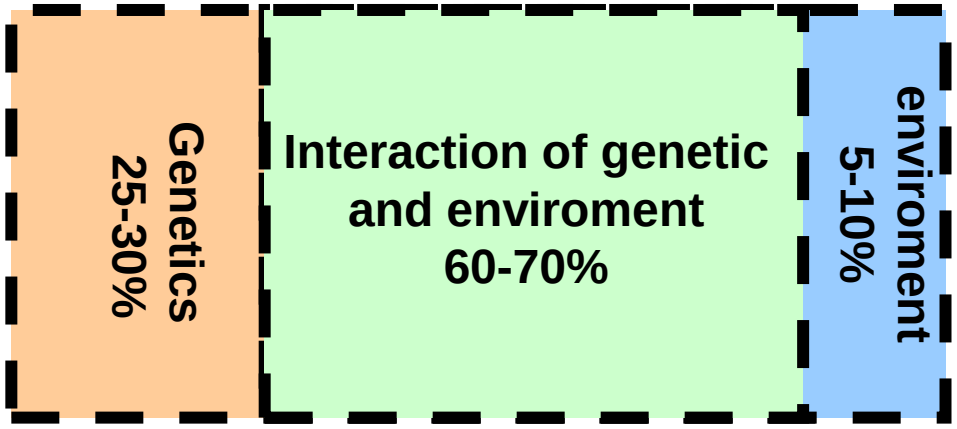
Neural tube defects (NTDs) are a group of disorders in which an opening in the spinal cord or brain remains from early in human development.



Failure of the tube to close properly can result in a number of NTDs .

Rank	1996			2000			2005			2006			2009		
	Birth defect	*n*	Prevalence	Birth defect	*n*	Prevalence	Birth defect	*n*	Prevalence	Birth defect	*n*	Prevalence	Birth defect	*n*	Prevalence
1	CL/P	611	14.46	CL/P	703	14.03	CHDs	1459	23.96	CHDs	1642	25.58	CHDs	3969	28.82
2	NTDs	574	13.59	PD	624	12.45	PD	893	14.66	PD	1016	15.83	PD	2191	15.91
3	PD	385	9.11	NTDs	599	11.95	CL/P	836	13.73	CL/P	914	14.24	CL/P	1814	13.17
4	CHC	271	6.41	CHDs	573	11.44	NTDs	538	8.84	NTDs	525	8.18	NTDs	892	6.48
5	CHDs	259	6.13	CHC	355	7.08	CHC	458	7.52	CHC	465	7.25	CHC	827	6.00

Neural Tube Defects—Complex diseases

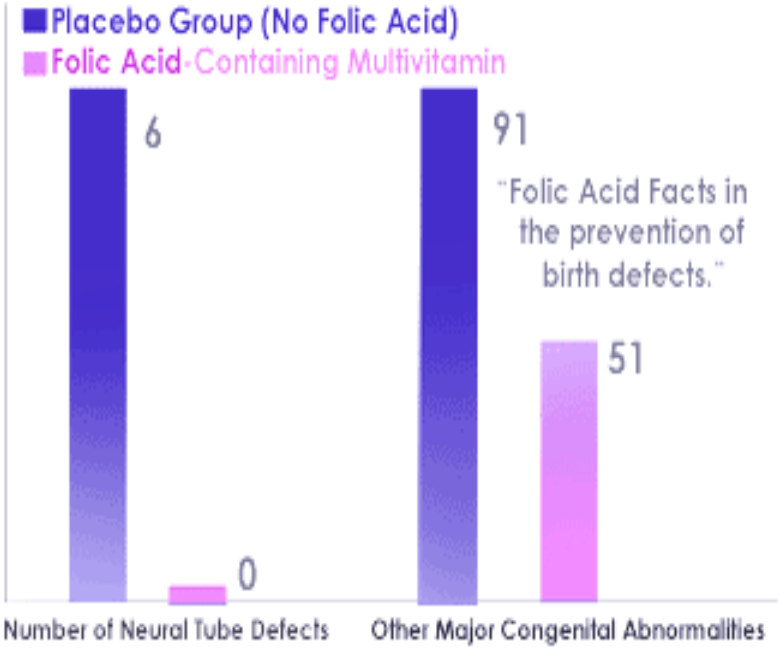


NTDs have several suspected causes and have been linked to genetic, ethnic, nutritional, drug, and environmental factors. It is probably the combination of several of these that produces an abnormality in any particular fetus.

Lower concentrations of folate and vitamin B-12 during pregnancy are higher risk factors of NTDs .

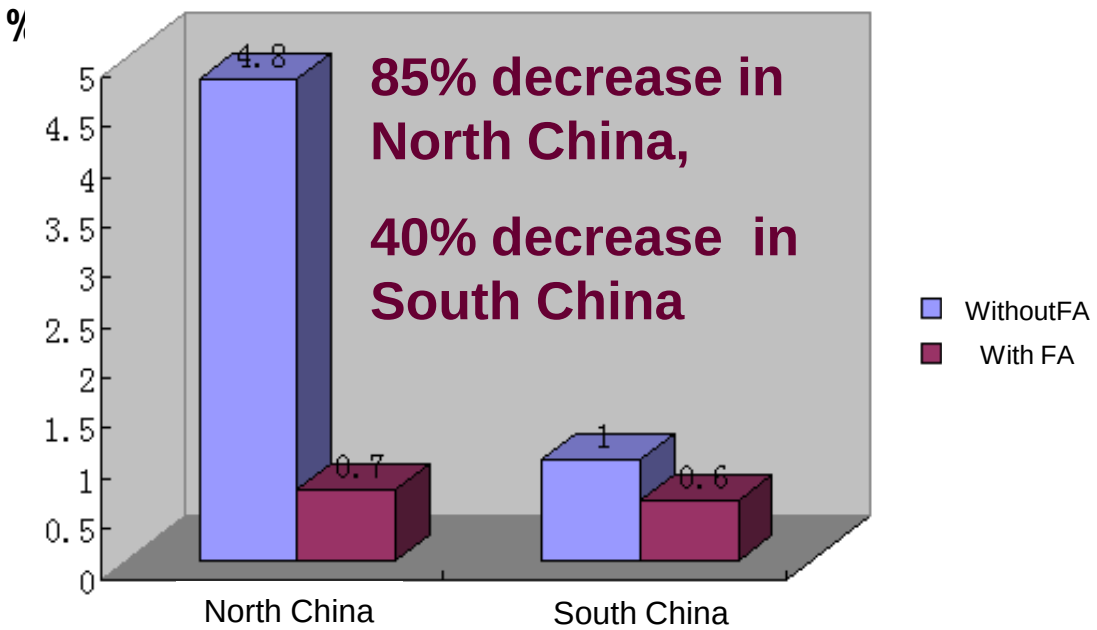
Preventive effect of folic acid on NTDs

The preventive effect of folic acid on NTD risk has been subjected to extensive epidemiologic research.



NEJM,2001

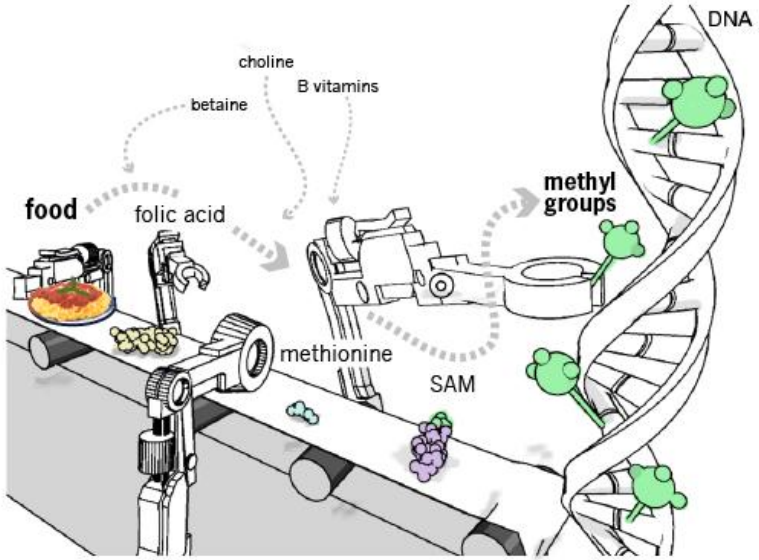
The preventive effect of folic acid on NTD risk in China



中华医学杂志，80：493，2000



Folic Acid, Methylation and NTDs



Intracellular folic acid and B vitamins are key players in providing an adequate source of methyl groups for methylation of DNA and proteins, in addition to their involvement in purine and pyrimidine metabolism.

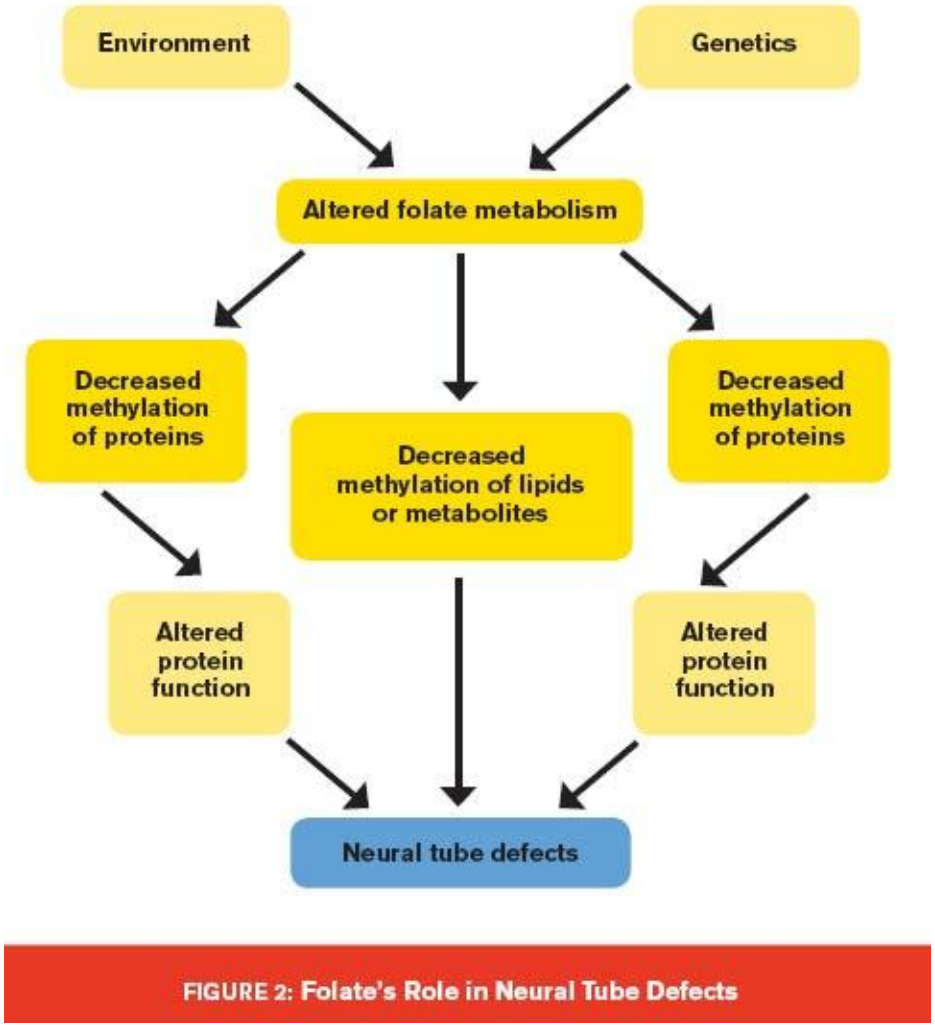


FIGURE 2: Folate's Role in Neural Tube Defects

Life Extension Magazine August 2009

Prevalence of NTDs in Shanxi province

Shanxi province:

BD (189.9/10,000),

NTDs (102.3/10,000)

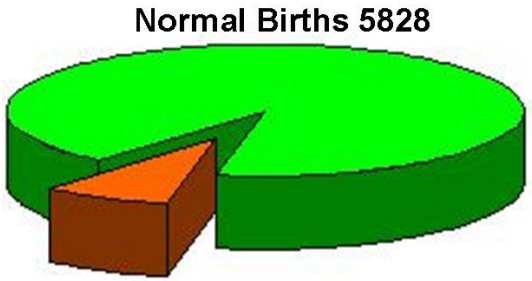
Lvliang mountain region:

BD (814.8/10,000),

NTDs (186.0/10,000)

------the highest area in Shanxi

6,345 infants and fetuses registered (include live births, stillbirths, medical terminations)



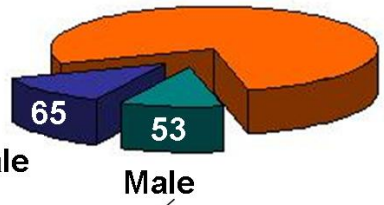
BD cases 517

Male:337;

Femal:179;

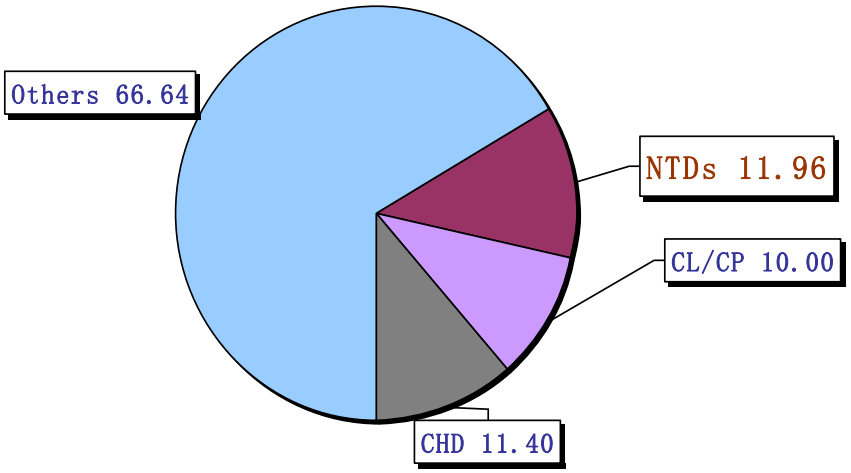
Unkown:1;

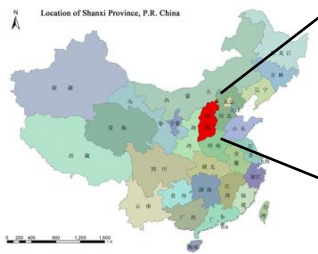
Pre: 814.8/10,000

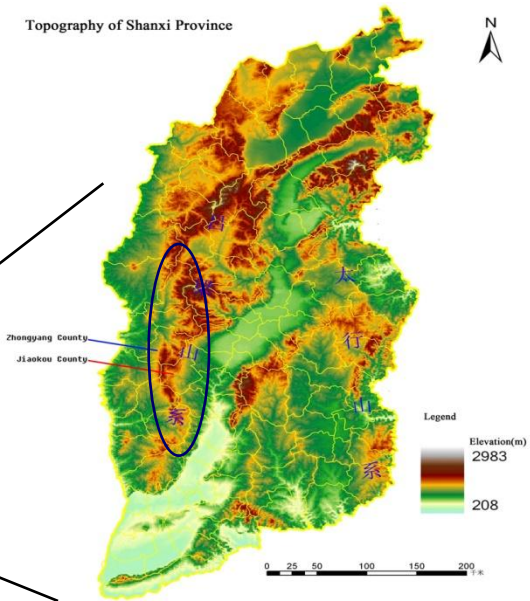


NTDs: 118

Pre: 186.0/10,000









Food consumption of residents and child bearing age women in two counties compared with national average

(g/reference/day)

Food	China(2002)		Resident Average	CBA Women Average
	Rural areas	Average		
Rice and Its Products	248.4	239.9	21.6	**25.1**
Wheat and Its Products	141.0	138.5	317.2	318.2
Other Cereals	25.9	23.3	63.2	63.3
Beans	4.8	4.2	4.2	4.2
Bean Products	11.4	11.8	1.1	1.1
Dark Green Vegetables	92.8	91.5	**26.8**	**29.6**
Green Vegetables	191.3	183.7	**117.6**	**127.9**
Pickles	11.0	10.3	1.0	1.1
Fruits	36.6	45.7	**21.0**	**17.2**
Nuts	3.3	3.9	0.5	0.3
Pork	47.8	51.3	**6.6**	**4.8**
Other Livestock Meat	6.8	9.2	**0.1**	**0.1**
Poultry Meat	11.1	14.3	**0.1**	**0.1**
Egg and Its Products	19.9	23.6	27.7	22.2
Fish and Shrimp	24.4	30.1	**0.1**	**0.0**
Vegetable Oil	29.9	32.7	18.3	19.2
Animal Fat	10.5	8.7	**0.4**	**0.4**



Dietary pattern and the risks of NTD incidence

Factors	Case (*n* 80)	Control (*n* 104)	OR†,‡	95 % CI
Meat (time(s)/week)				
≥3	9	32		1·00 referent
1–2	12	30	1·42	0·53, 3·86
<1	59	42	5·00	2·16, 11·56
Egg or milk (time(s)/week)				
≥5	3	19		1·00 referent
≥3	23	41	3·55	0·95, 13·31
1–2	21	19	7·00	1·79, 27·46
<1	33	25	8·36	2·23, 31·42
Fresh vegetables (time(s)/week)				
≥5	24	68		1·00 referent
≥3	19	26	2·07	0·98, 4·40
1–2	18	5	10·20	3·41, 30·48
<1	19	5	10·7	3·62, 32·01
Fruits (time(s)/week)				
≥5	11	62		1·00 referent
≥3	14	20	3·95	1·55, 10·07
1–2	16	9	10·02	3·55, 28·30
<1	39	13	16·91	6·89, 41·48
Pickled vegetable (time(s)/week)				
<1	73	86		1·00 referent
≥1	7	18	0·46	0·18, 1·16
Sprouted potato (time(s)/week)				
<1	55	95		1·00 referent
≥1	25	9	4·798	2·09, 11·02



functional shortage of one-carbon nutrients

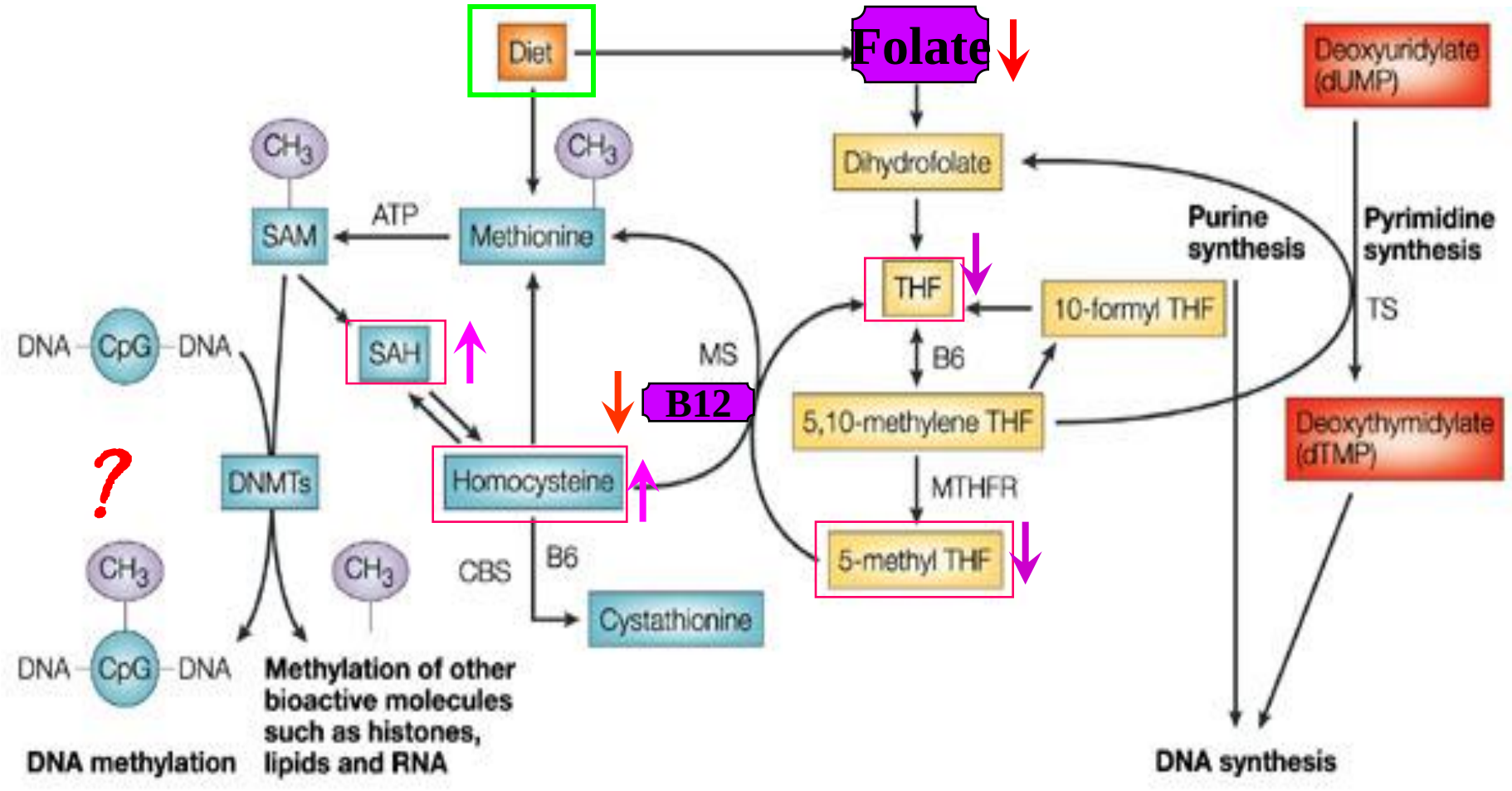
Crude and adjusted *P* values for the comparison of natural log-transformed serum component concentrations between cases and controls

Components and ratio[1]	Cases (n = 46) $\bar{X} \pm SD$	Controls (n = 50) $\bar{X} \pm SD$	P^2	Adjusted P^3
5-MeTHF (nmol/L)	9.32 (4.52)	14.58 (9.06)	<0.001	<0.001
5-FoTHF (nmol/L)	2.64 (1.59)	4.35 (2.60)	<0.001	0.002
Folic acid (nmol/L)	1.56 (3.15)	1.39 (2.93)	0.798	0.728
Serine (μmol/L)	1.30 (1.33)	1.03 (1.05)	0.239	0.188
Histidine (μmol/L)	19.55 (8.27)	19.63 (7.36)	0.949	0.999
Homocysteine (μmol/L)	8.14 (4.38)	6.92 (3.72)	0.159	0.491
Cystathionine (nmol/L)	8.60 (6.46)	7.20 (6.94)	0.194	0.284
Methionine (μmol/L)	10.34 (9.00)	13.80 (12.44)	0.330	0.338
SAM (nmol/L)	47.76 (24.70)	49.41 (19.43)	0.342	0.317
SAH (nmol/L)	12.60 (7.58)	9.25 (4.13)	0.017	0.018
SAM:SAH	5.51 (4.32)	7.60 (6.61)	0.025	0.025
Total folate (nmol/L)	9.96 (3.90)	16.07 (9.00)	<0.001	<0.001
Vitamin B12 (pmol/L)	83.24 (29.42)	109.81 (49.41)	0.002	0.003

ExpNeurol 2008;212:515–21.



functional shortage of one-carbon nutrition



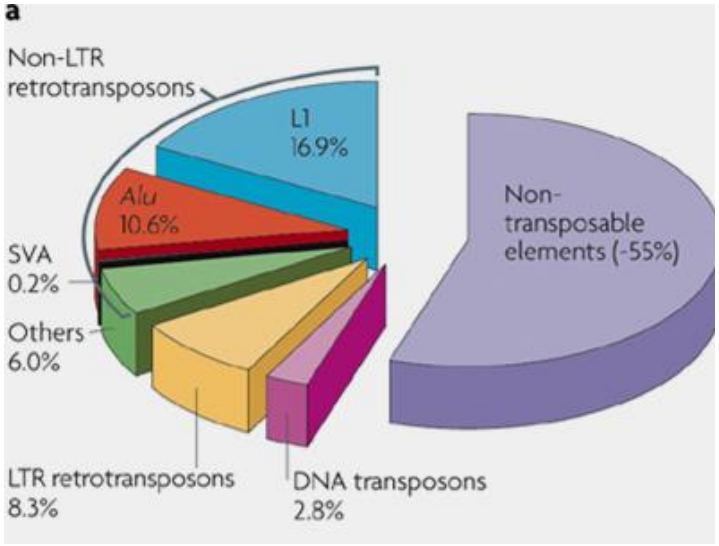
One carbon unit metabolism in association with Genomic regulative events

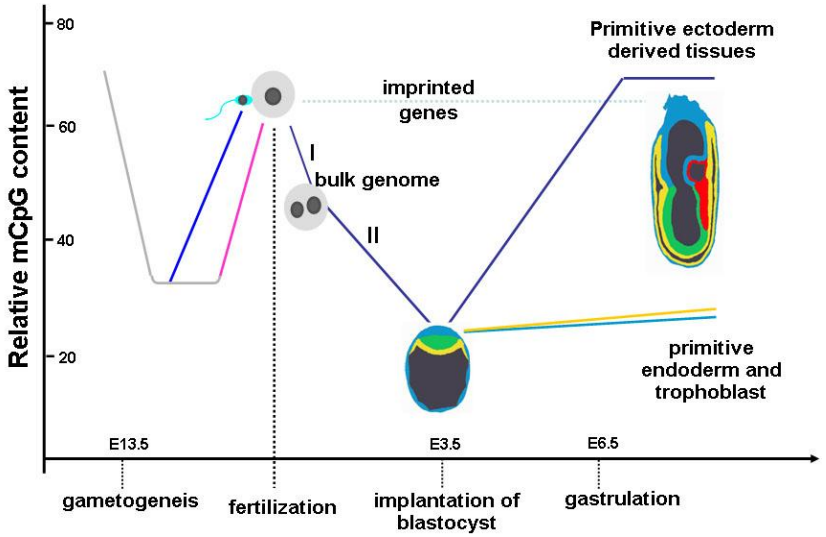
Nature Reviews | Cancer

Methylation modification and NTDs

Transposons and **imprinted genes** are 2 classes of elements within the human genome that have methylation patterns that are sensitive to malnutrition in early embryonic development.

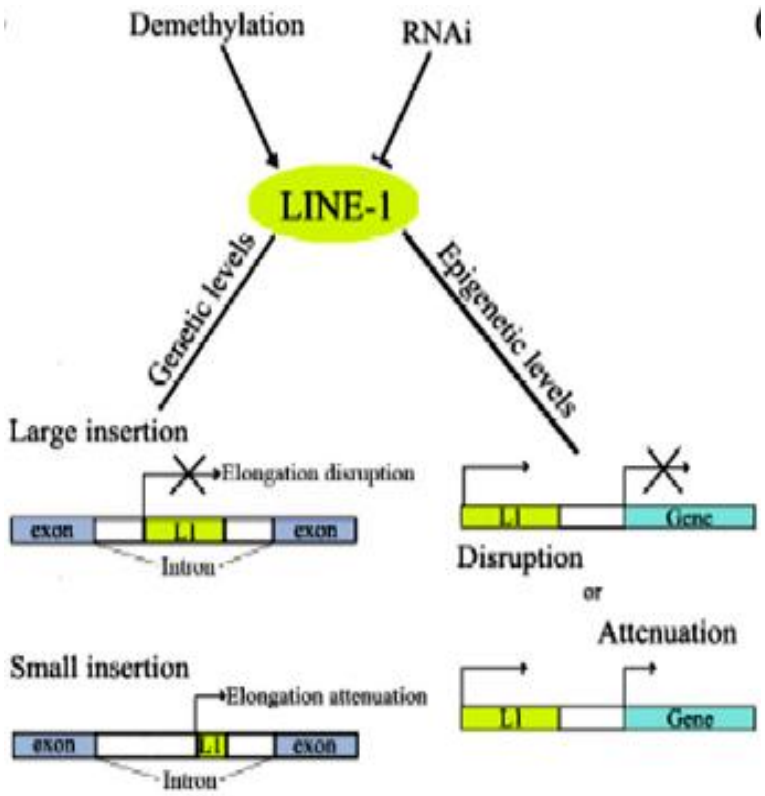
—*Nutrition 2004;20:63–8.*





Nature Review

long interspersed nucleotide element-1



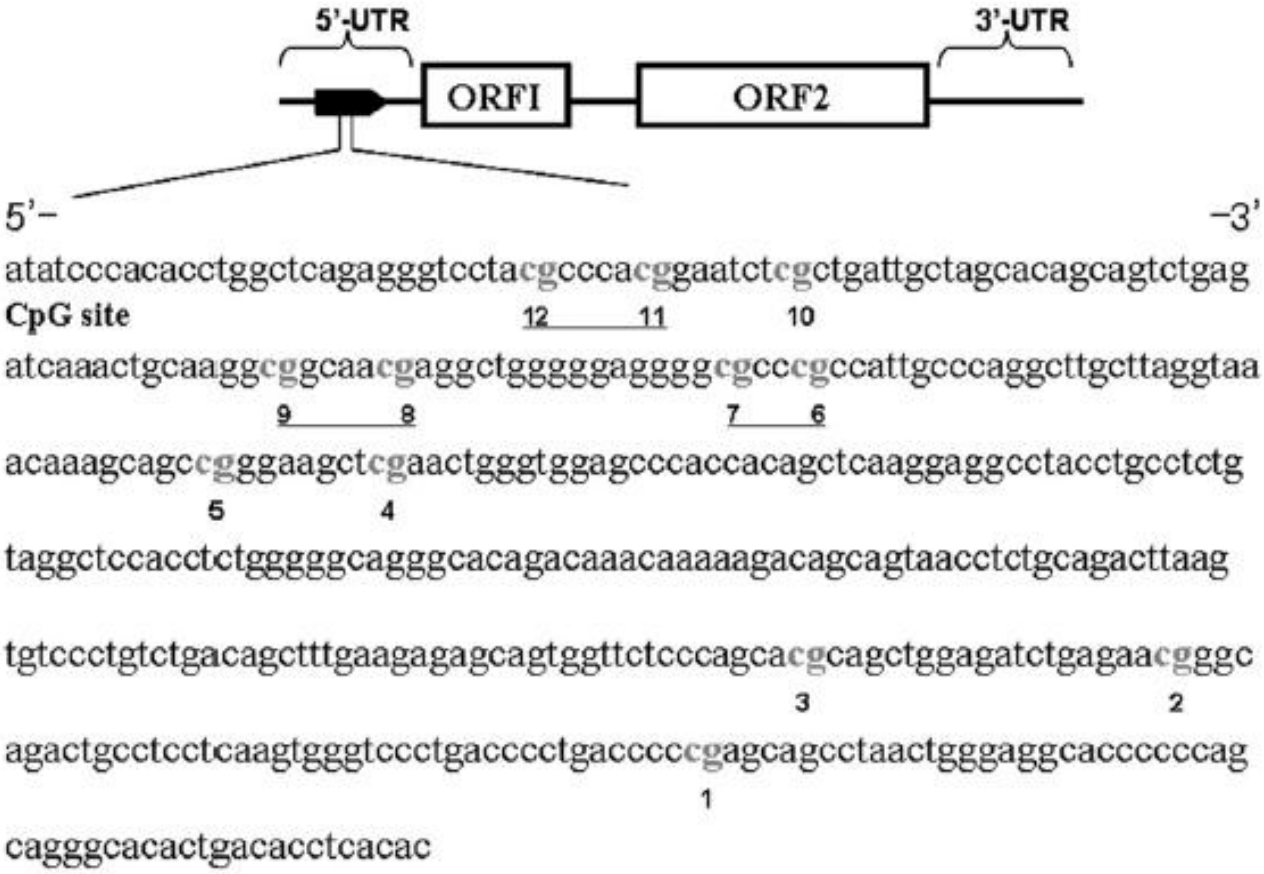
LINE-1 retrotransposon is regulated by epigenetic mechanisms.

LINE-1 elements are normally silenced by hypermethylation.

Activation of LINE-1 retrotransposition can affect own stream gene expression.

Hypomethylation of LINE-1 in some cancers is thought to be connected to genomic instability and endogenous DNA doublestrand breaks.

The structure of LINE-1



Schematic structure of the LINE-1 element

- *Am J Clin Nutr 2010;91:1359–67.*



DNA hypomethylation in NTDs

Methylation level of LINE-1 and genomic DNA between cephalic malformations, spina bifida and control group (Mean±SD)

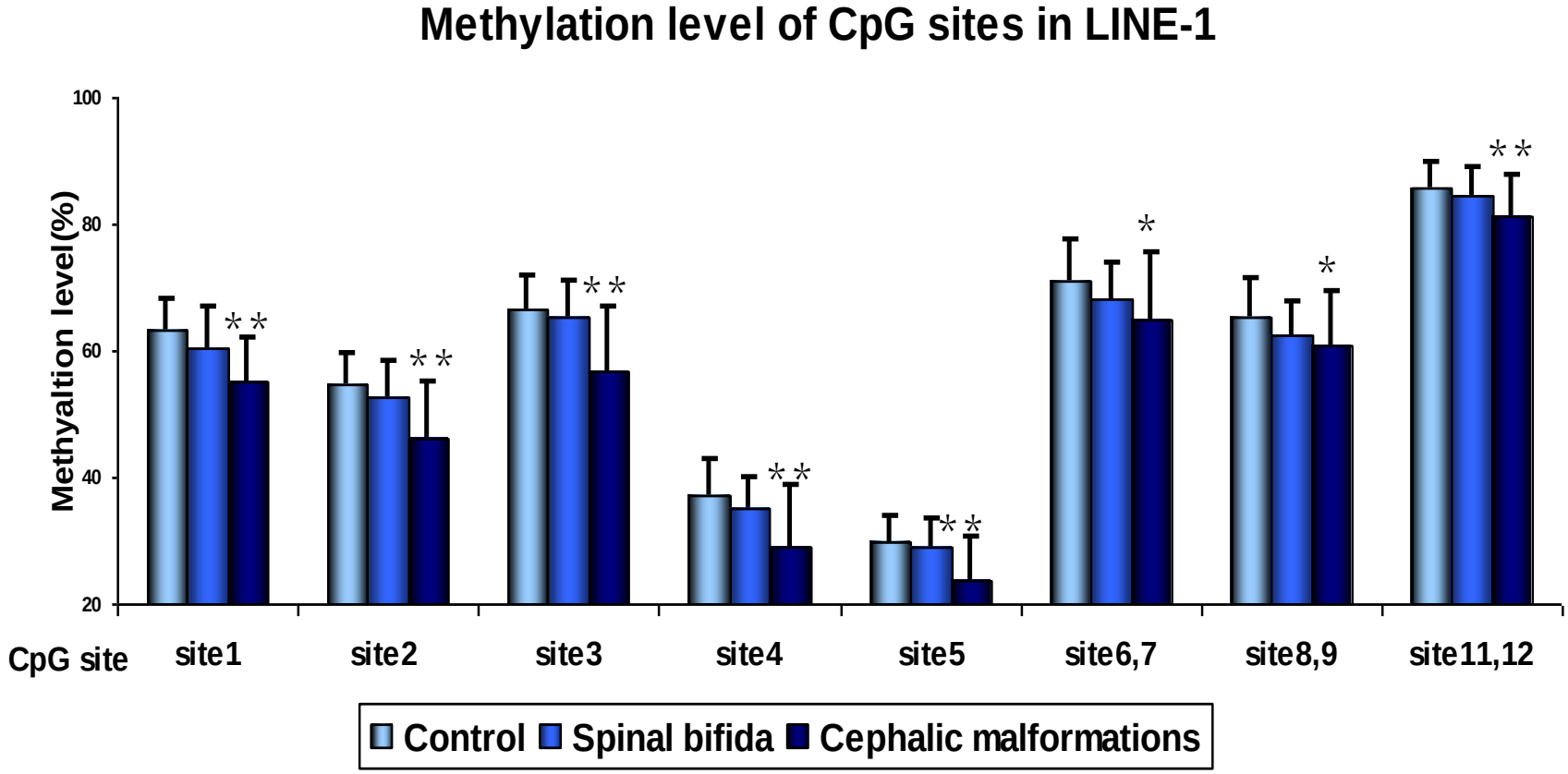
	Line-1 methylation level(%)	Global methylation level(%)
NTDs(n=48)	54.57±7.63**	5.01±2.12*†
Cephalic malformations(n=27)	**52.43±8.84****	**4.79±2.27§**
spina bifida(n=21)	57.32±4.60	5.26±1.98
Controls(n=49)	**59.03±4.43**	**5.95±2.38**

n: sample numbers †n=45 §n=24

****p<0.001 * p<0.05**

- *Am J Clin Nutr 2010;91:1359–67.*

DNA hypomethylation in NTDs



- *Am J Clin Nutr 2010;91:1359–67.*



Assessment of risk of NTDs

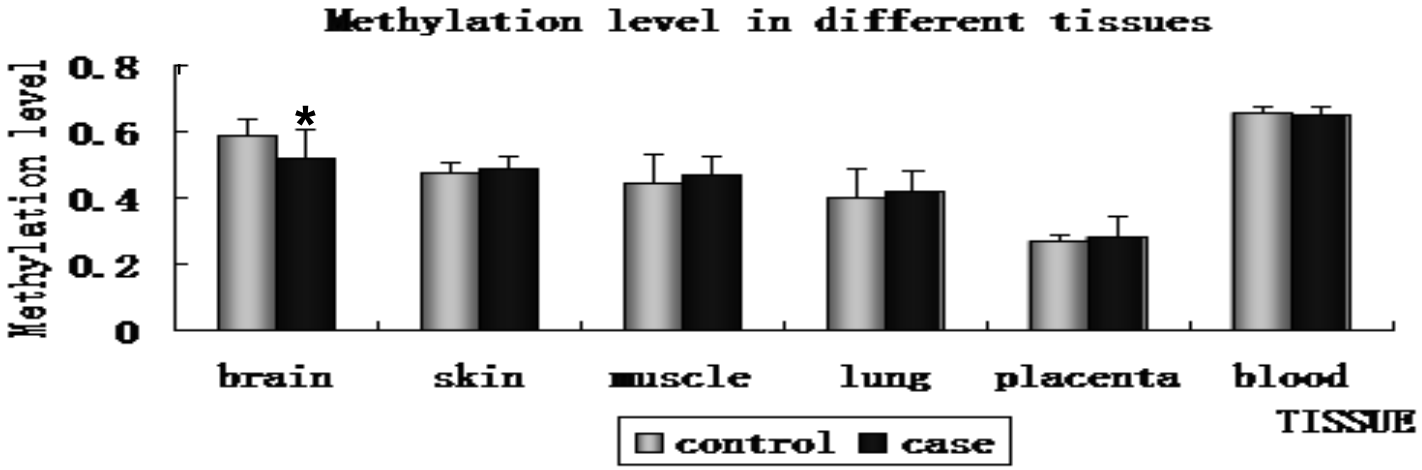
NTDs risks assessments with methylation level of genomic DNA and LINE-1

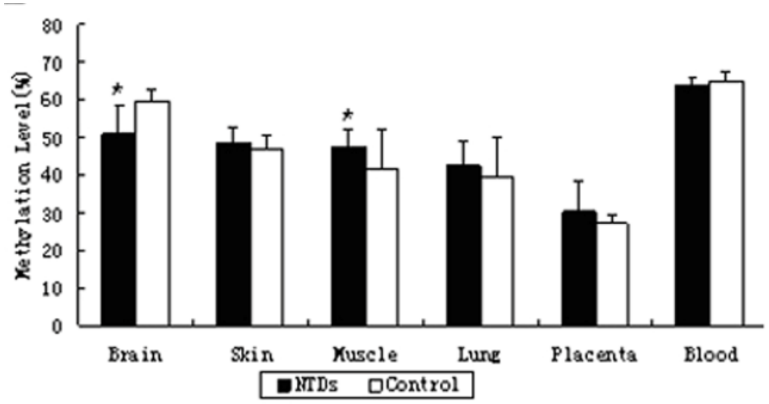
	NTDs n(%)	Control n(%)	OR (95% CI)	Adjusted OR§ (95% CI)
LINE-1 methyaltion level	48	49		
≥75%	5(10.4%)	11(22.5%)	1(reference)	1(reference)
25%~75%	12(25%)	25(51.0%)	1.056 (0.299-3.728)	0.727 (0.183-2.889)
≤25%	31(64.6%)	13(26.5%)	**5.246* (1.519-18.124)**	**5.966* (1.568-22.704)**
Global methyaltion	45	49		
≥75%	8(17.8%)	13(26.5%)	1(reference)	1(reference)
25%~75%	17(37.8%)	23(47.0%)	1.201 (0.407-3.541)	0.949 (0.293-3.078)
≤25%	20(44.4%)	13(26.5%)	2.500 (0.812-7.692)	2.931 (0.870-9.872)

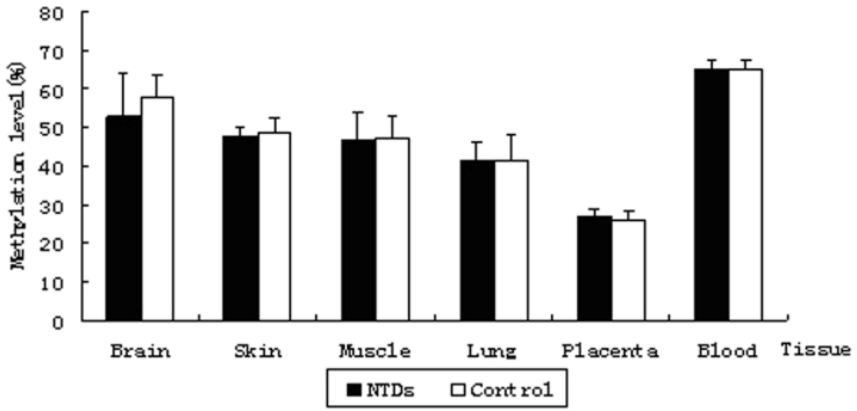
NTDs – neural tube defects; OR: odds ratio; CI: confidence interval
*p<0.01；
Defined 25and75 percentile of control group methylation level as cutoff value
§ Adjusted with sex and gestation week

- *Am J Clin Nutr 2010;91:1359–67.*

Methylation changes in tissues



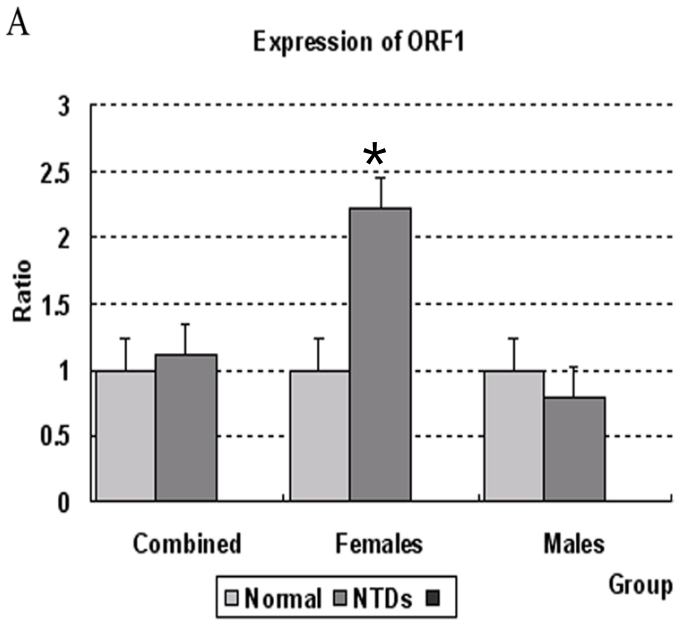


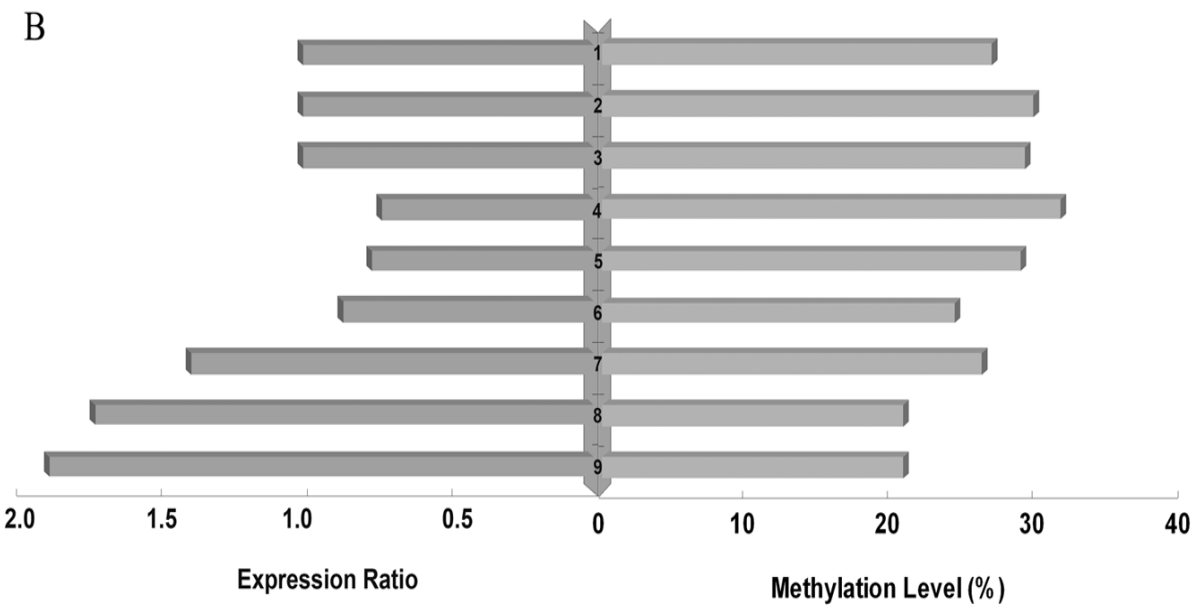


Difference in L1Hs methylation between genders.



ORF1p expression and methylation





- Increased expression of ORF1p was detected in samples from female NTDs brain tissues .
- ORF1p expression increased with decreasing level of L1Hs methylation (r=1.61, p=0.006, in HCT-15 cells with 5-Aza treatment: 0μm (Lines 1-3), 15μm (Lines 4-6), 50μm (Lines 7-9))

DNA damage and chr. accessibility

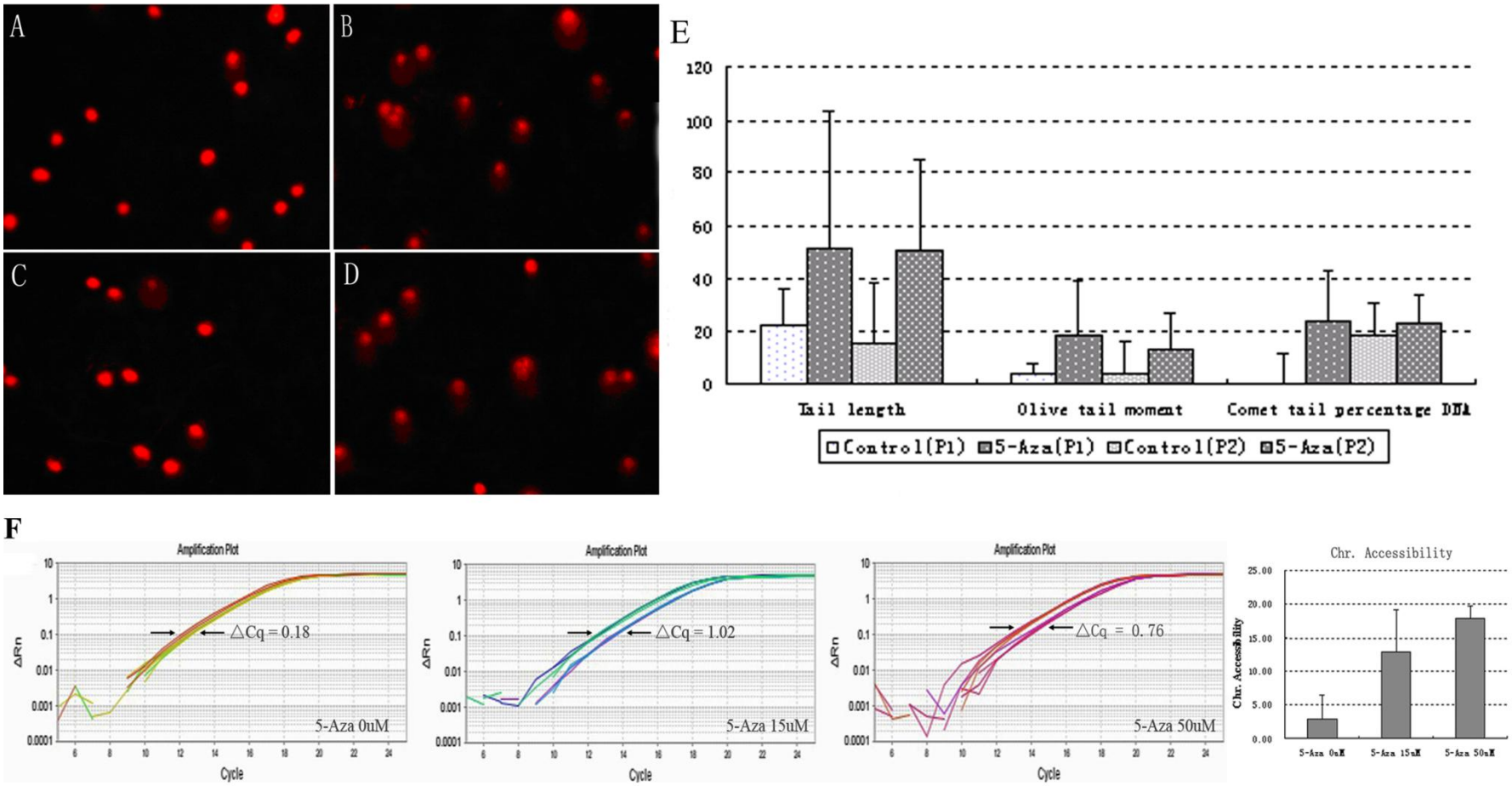


Figure 5 DNA damage and chromatin accessibility in HCT15 treated with 5-Aza



methylation level and maternal nutrition

Comparison of maternal vitamin B12, folate and tHcy between NTDs and control group (Mean ± SD)

	NTDs(*n*=32)	Controls(*n*=36)	p-value
vitamin B_{12} (pmol L^{-1})	70.16 ± 19.03	88.62 ± 38.92	0.015
Folate (nmol L^{-1})	8.38 ± 3.23	10.57 ± 8.83	0.172
tHcy (μmol L^{-1})	15.21 ± 7.30†	12.09 ± 7.33	0.068

Correlations between methylation level and maternal nutrition†

	Pearson correlation	
	LINE-1 methylation	**Global methylation**
Folate (case/control)	0.026 (36/32)	0.147 (28/30)
vitamin B_{12}(case/control)	0.047 (36/32)	0.073 (28/30)
tHcy(case/control)	0.015 (36/29)	0.147 (36/29)

† Correlation was performed including all NTDs and controls, similar results were obtained when correlation was performed in cases and controls respectively

- *Am J Clin Nutr 2010;91:1359–67.*



Summary 1

✓**Hypomethylation of LINE-1 and genomic DNA was associated with an increased risk of NTDs.**

✓**LINE-1 hypomethylation in NTDs was associated with a significant increase in expression level, DNA instability and chromatins accessibility.**

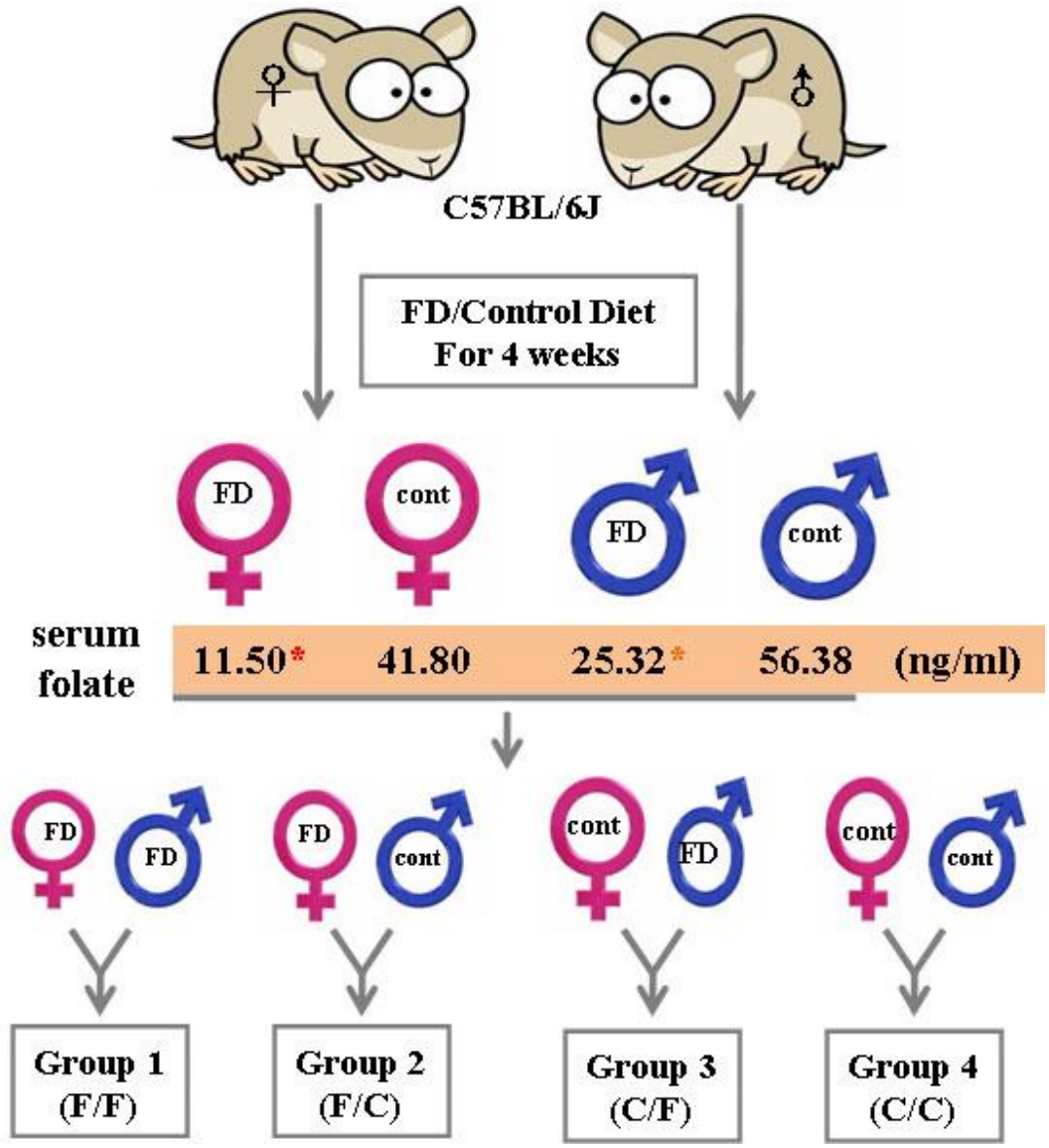
✓**Functional insufficiency of maternal plasma vitamin B-12 and folate were associated with NTDs, although no significant correlation established between maternal nutrition and LINE-1 methylation.**

----the target genes? that folate regulated through methylation modifications and increase risk of NTD

Mice and diets

Diet formulation based on the AIN-93G （g/kg）

Ingredient	Control	FD
Cornstarch	380.500	380.500
Amino acids mix *	200.000	200.000
Dextrinized cornstarch	152.000	132.000
Sucrose	100.000	100.000
Soybean oil	70.000	70.000
Fiber	50.000	50.000
Mineral mix *	35.000	35.000
Vitamin Mix *	10.000	\
w/o Folate Vitamin Mix *	\	10.000
Choline bitartrate	2.500	2.500



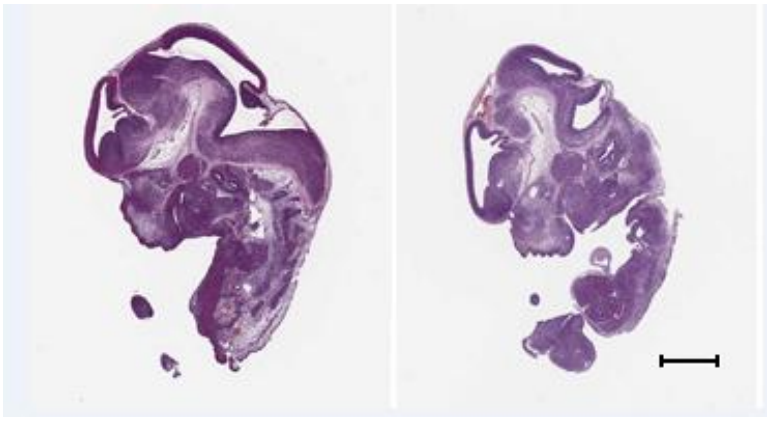


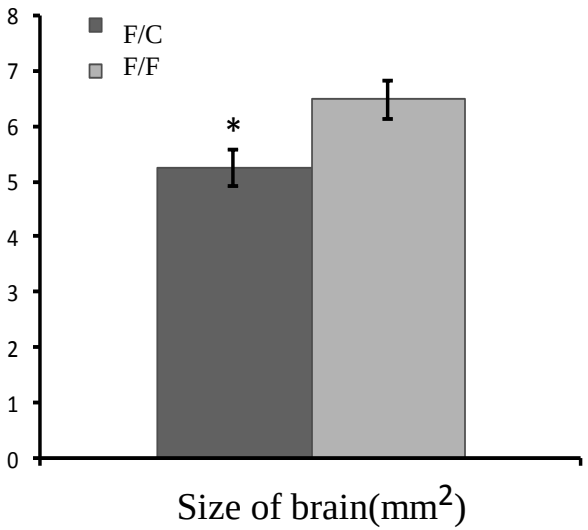
Reproductive outcomes

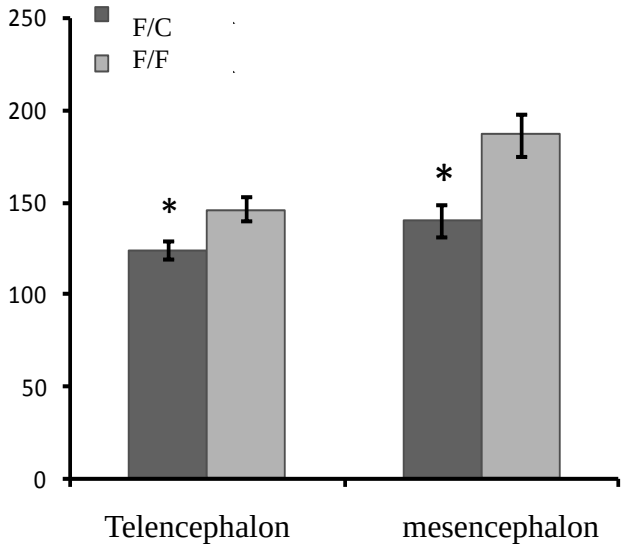
Group (n)	Maternal Weight	No. of Fetus	Ratio of resorpted fetus(%)	Crown-rump Length(cm)	Weight of Fetus (mg)	Weight of Placenta (mg)
Group 1 (F/F)(17)	36.9±6.0	8.1±2.8	8.2±13.2	2.4±0.1	1173±70	106±217
Group 2 (C/F)(9)	35.8±4.6	6.0±3.1	22.0±34.5	2.3±0.1	1300±101	120±20
Group3 (F/C)(16)	35.3±4.9	7.1±2.3	4.4±8.6	2.0±0.1	1214±112	113±30
Group 4 (C/C)(13)	34.1±4.9	7.2±2.4	3.2±6.0	2.4±0.2	1241±119	100±24

Developmental retardation of brain











Rare developmental malformations

No.	Group	Number of fetus/resorption	Phenotype	Picture
1	Group 1 (F/F)	11/2	anophthalmia	
2	Group 1 (F/F)	10/0	spinal bending	
3	Group 1 (F/F)	10/0	thinness， suspected spina bifida	
4	Group 1 (F/F)	8/1	spin tail	
5	Group 2 (C/F)	9/0	anophthalmia	
6	Group 2 (C/F)	8/4	microphthalmus	
7	Group 2 (C/F)	9/1	coil tail	
8	Group3 (F/C)	7/1	suspected spina bifida	
9	Group3 (F/C)	7/0	suspected spina bifida	
10	Group3 (F/C)	9/0	stillborn foetus	

Rare developmental malformations

F/F group



F/C group



C/F group





imprinting regulation

Group (n)	Maternal Weight	No. of Fetus	Ratio of absorbed fetus(%)	Crown-rump Length(cm)	Weight of Fetus (mg)	Weight of Placenta (mg)
Group 1 (F/F)(17)	36.89±5.91	8.1±2.78	8.16±13.21	2.35±0.13	1173.3±70.8	106.3±216.9
Group 2 (C/F)(9)	35.77±4.60	6.00±3.12	22.00±34.52	2.25±0.11	1300.0±101.2	120.0±20.0
Group3 (F/C)(16)	35.33±4.95	7.06±2.26	4.42±8.60	2.07±0.10	1214.1±112.2	112.9±29.9
Group 4 (C/C)(13)	34.05±4.97	7.17±2.44	3.16±6.02	2.37±0.22	1241.3±119.2	100.9±24.4

Paternal folate deficiency showed limitation of growth, such as decrease of fetus weight or length; while maternal folate deficiency decreased live birth, instead of size of fetus.

------ consistent with imprinting regulation?

- The father's genes that encode for imprinting tend to be growth limiting;
- The mother‘s is often to conserve resources for her own and current and subsequent litters



Gnas imprinting and NTDs

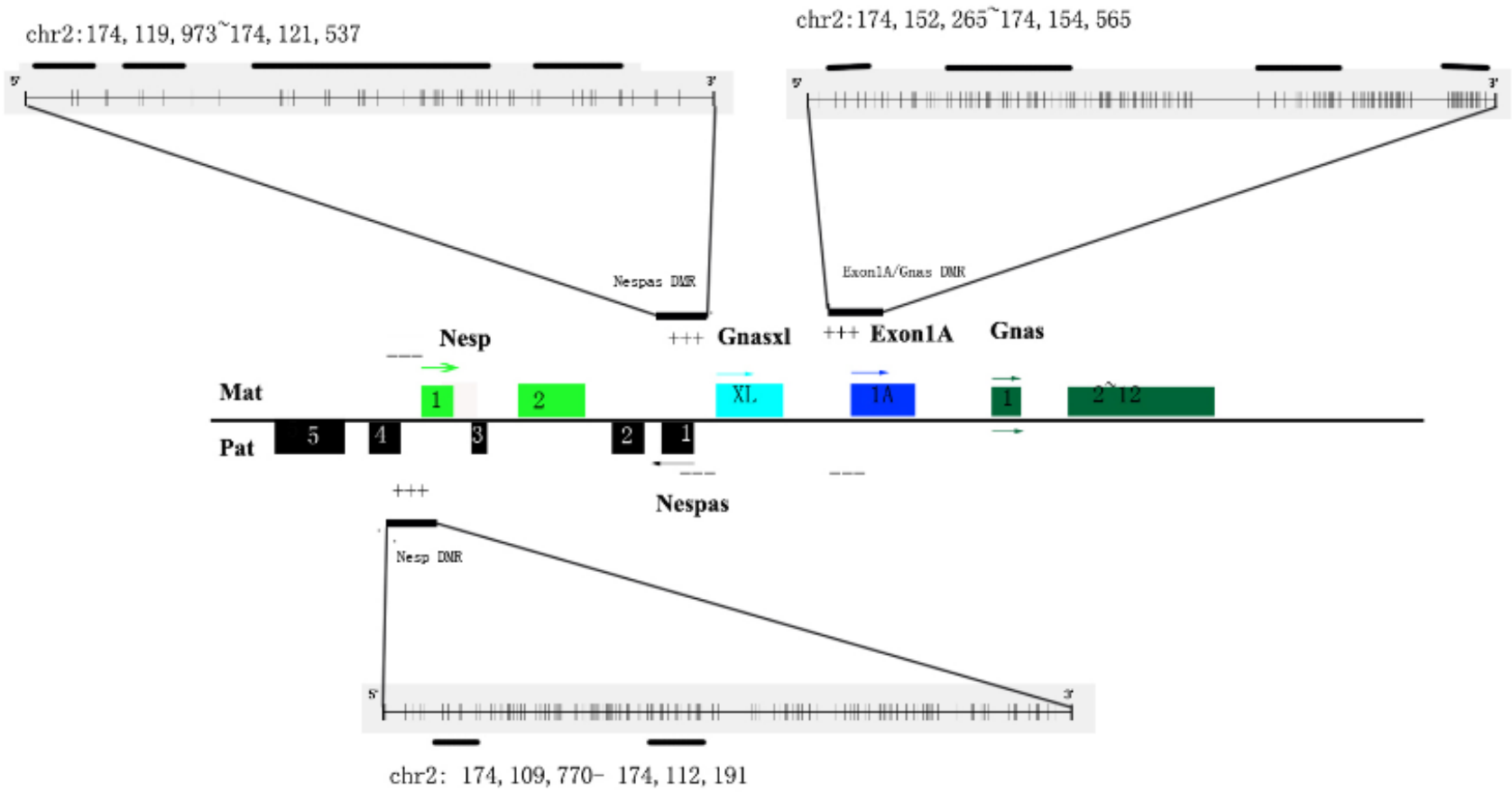
Gnas imprinting cluster :

- Gnas encodes the alpha subunit of a major heterotrimeric Gs signaling protein, which takes part in fetal growth and development.

- Gene expression studies in IUGR placentas have demonstrated the downregulation of Gnas.

Three differentially methylated regions (DMRs) have been found in the Gnas imprinting cluster, two of which are gDMRs. The difference of DNA methylation on gDMRs is established during gametogenesis and maintained throughout development.

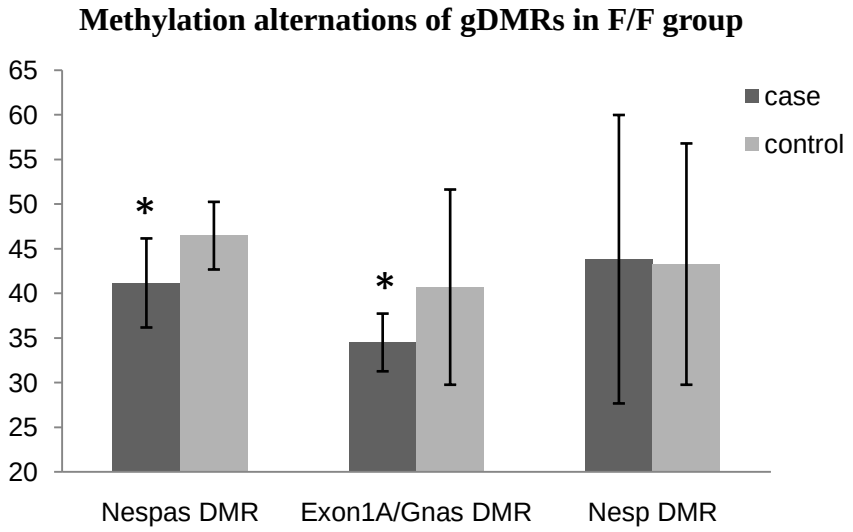
Structure of Gnas imprinting cluster

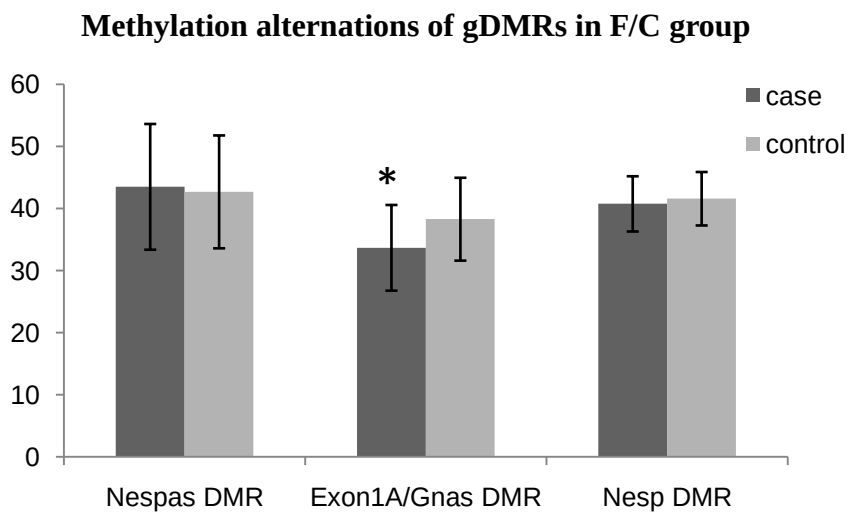


Three differentially methylated regions (DMRs) in the Gnas imprinting cluster:

- germline maternally methylated region at the Nespas promoter – Nespas gDMR;
- germline maternally methylated region at the Exon1A promoter – Exon1A/Gnas gDMR;
- paternally methylated region spanning the Nesp promoter – Nesp DMR.

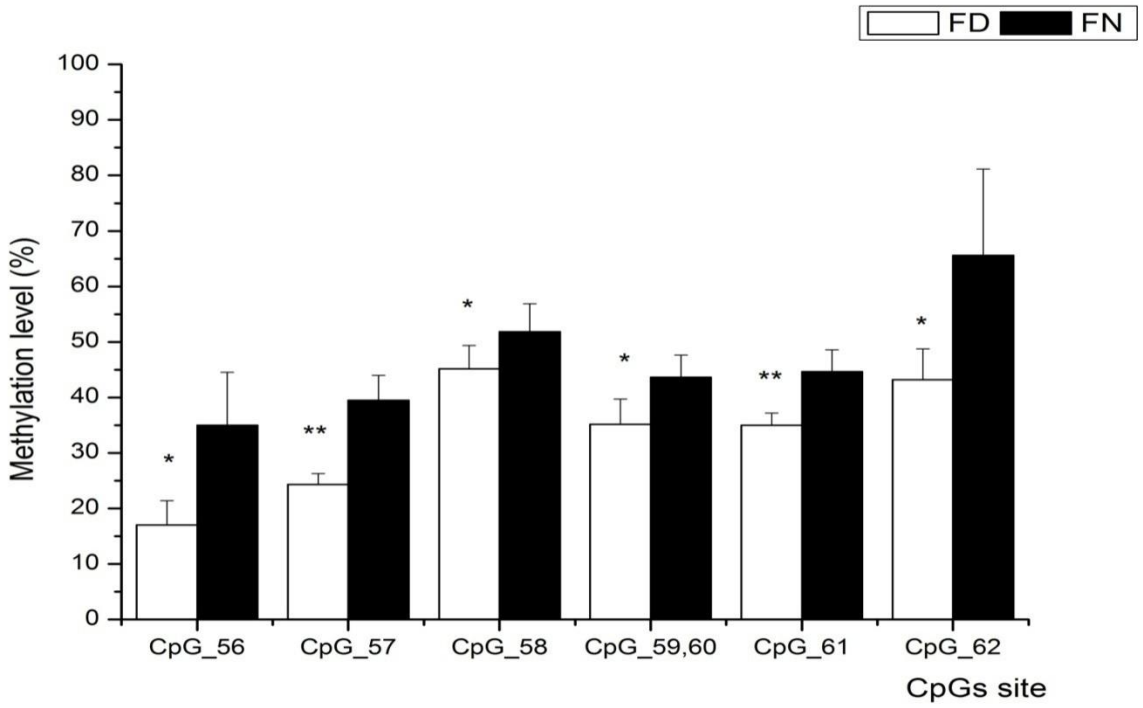
Imprinting aberrant in F/F & F/C fetus





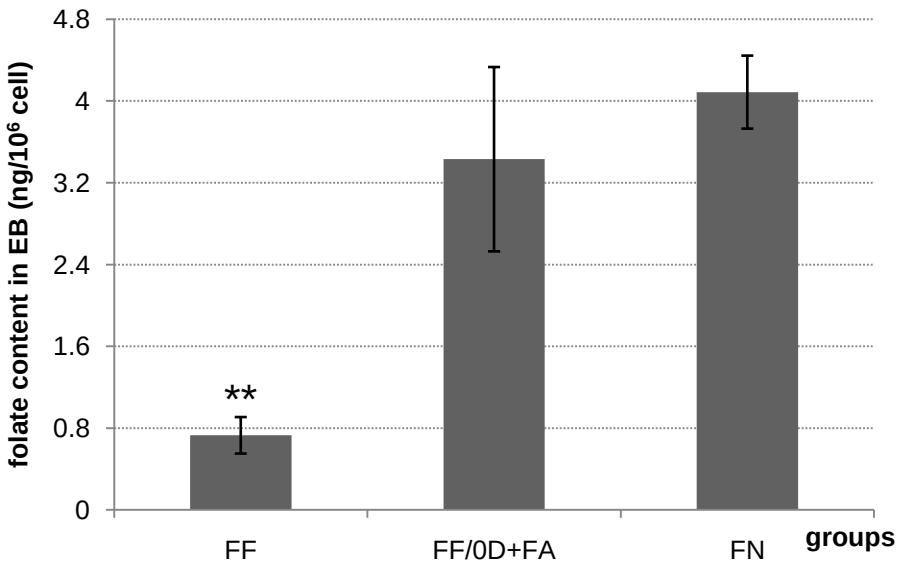
	Nespas DMR (Mean±SD, %)	Exon1A/Gnas DMR (Mean±SD, %)	Nesp DMR (Mean±SD, %)
Group 1 (F/F)	41.16±4.99	34.50±3.23	43.82±16.15
Control (C/C)	46.46±3.79	40.70±10.93	43.28±13.51
p	0.030	0.001	0.81
Group 2 (F/C)	43.49±10.12	33.67±6.90	40.75±4.44
Control (C/C)	42.68±9.09	38.28±6.67	41.58±4.30
p	0.544	0.013	0.708

Imprinting within Nespas DMR in F/C fetus



Methylation level of CpG Unit in chr2:174,120,698–174,120,842 of Nespas DMR region

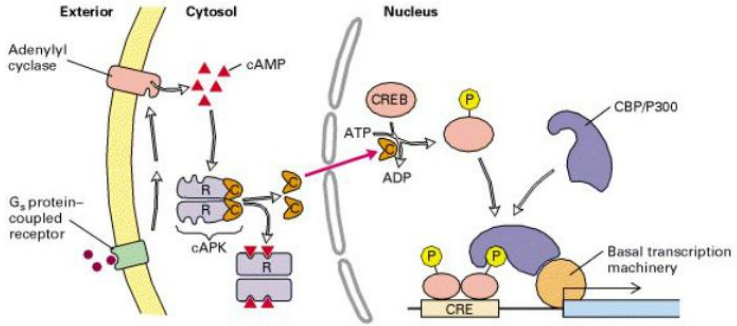
Folate deficiency directly induced loss of imprinting

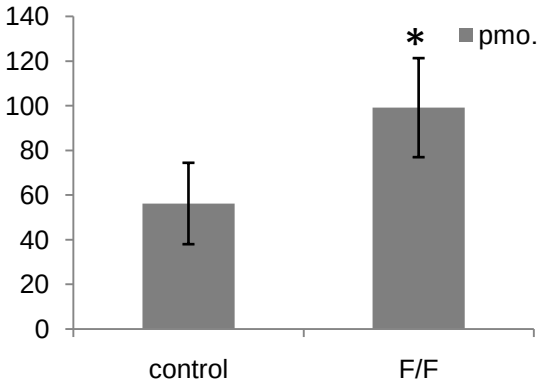


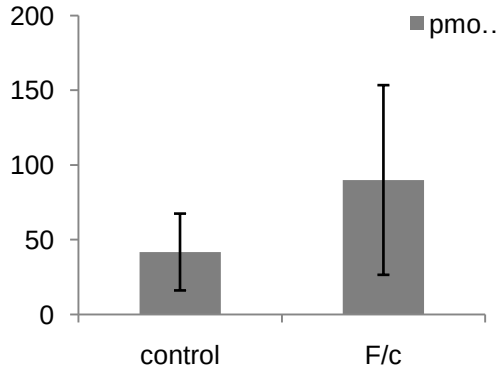
	Nespase DMR (%)	Exon1A/Gnas DMR (%)	Nesp DMR (%)
FF	30.5±0.5*	13.5±1.4*	12.1±2.4*
FN	38.8±2.3	19.2±1.6	17.7±0.8

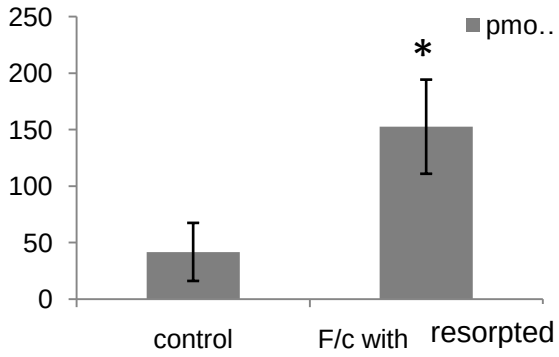


cAMP levels in fetus brain

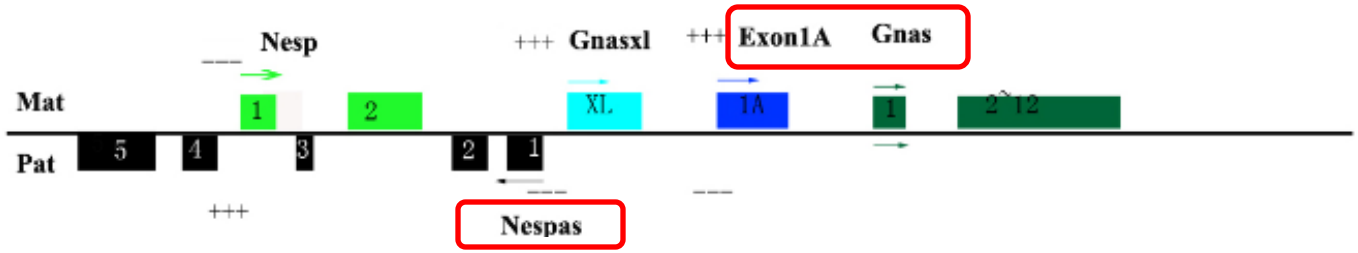


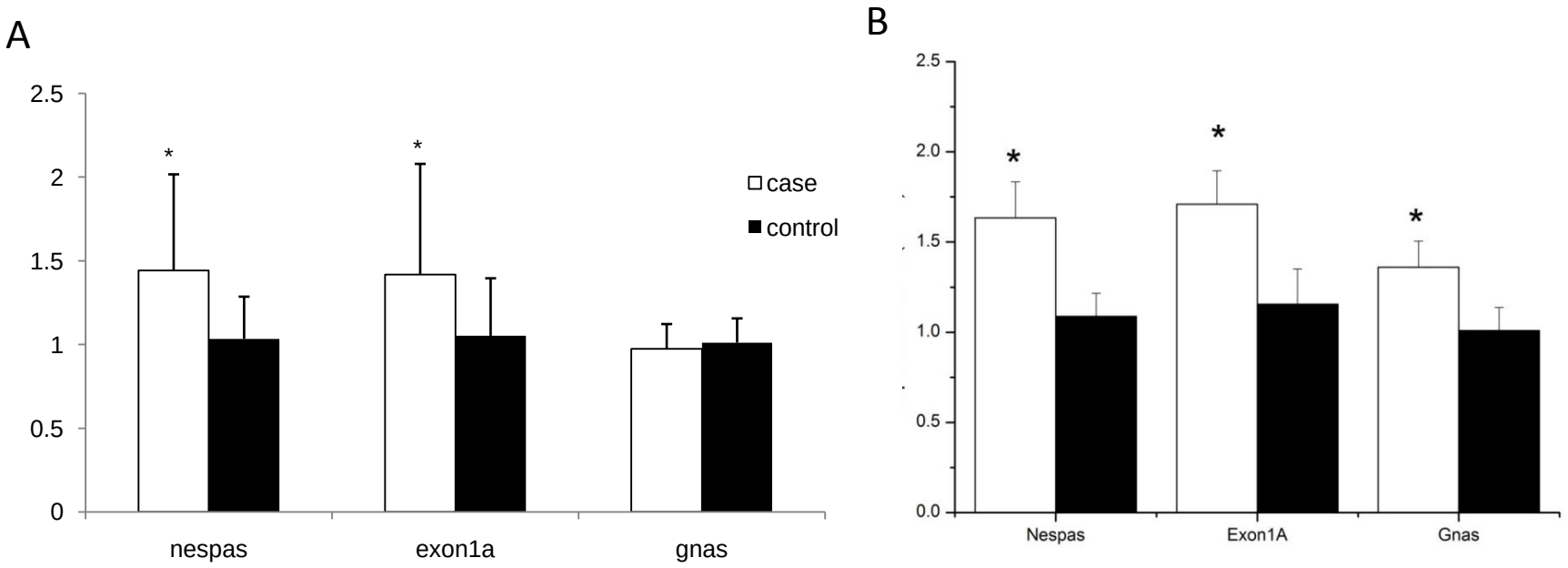






Gene expression in imprinting clusters





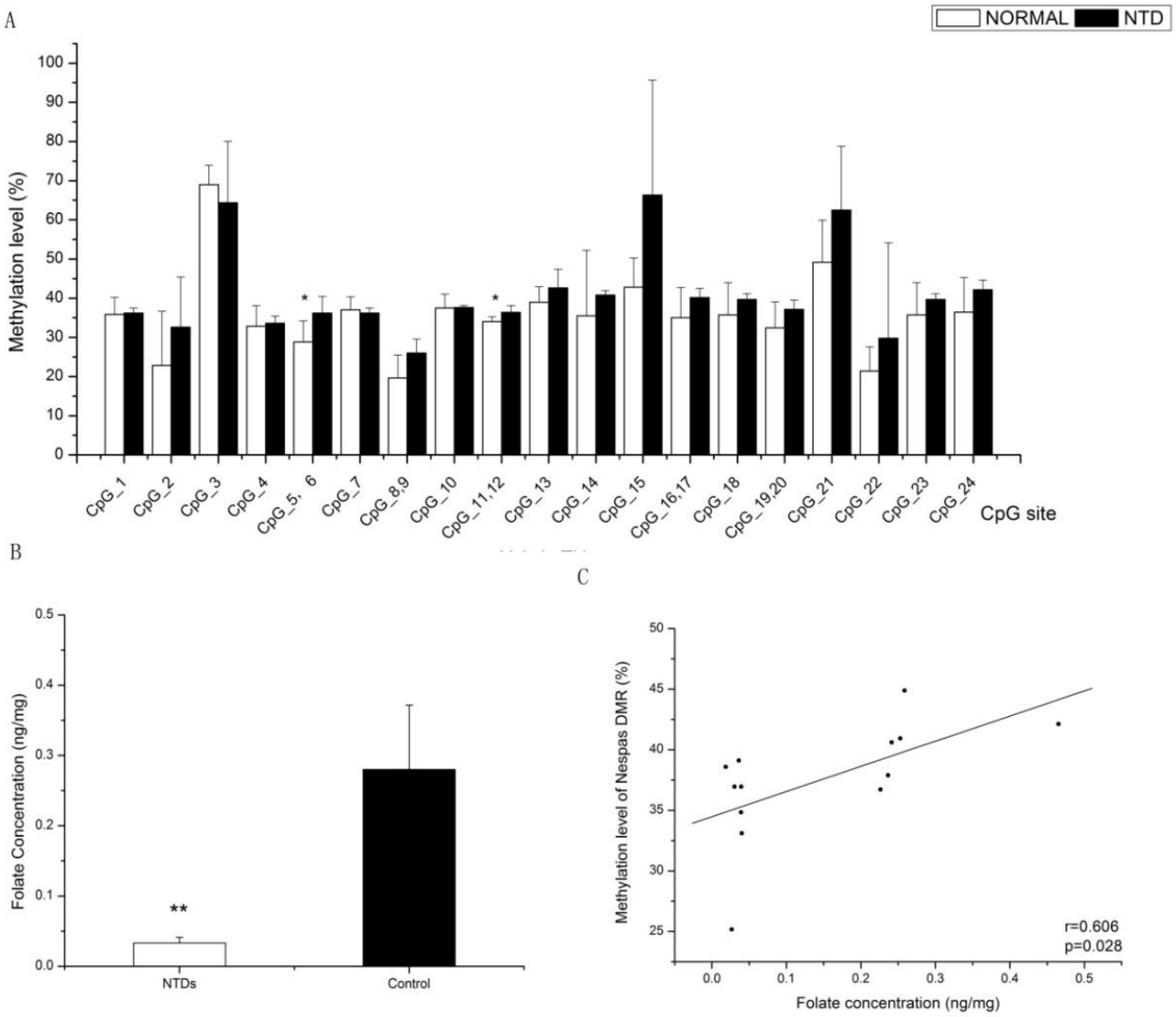
A: in brain tissue of F/F group; B: in brain tissue of F/C group



GNAS Imprinting in NTDs with low folate level

Sample	Phenotype	Folate level (ng/mg)	gDMR Methyaltion Level (%)1		
			GNAS-AS1	EXON1A/GNAS	NESP
NTD 1	Bifid spine; Renal fusion; Talipes Equinovarus; Hydrocephaly	0.02	38.58	45.50	44.80
NTD 2	Bifid spine; Hydrocephaly; Talipes Equinovarus	0.03	25.17	35.50	37.00
NTD 3	Anencephalus; Occipital meningeal encephalocele; IUGR	0.03	36.95	37.50	39.13
NTD 4	Bifid spine; Unilateral agenesis of kidney and ureter; Talipes Equinovarus	0.04	39.11	42.00	43.60
NTD 5	Occipital meningeal encephalocele	0.04	34.83	39.50	37.93
NTD 6	Bifid spine; Hydrocephaly; Congenital heart disease	0.04	33.11	38.00	36.93
Control 1	No malformation	0.23	36.72	39.00	43.21
Control 2	No malformation	0.24	37.89	39.50	32.73
Control 3	No malformation; Abdominal hemorrhagic effusion	0.24	40.61	39.00	33.78
Control 4	No malformation	0.25	40.94	37.50	35.13
Control 5	No malformation	0.26	44.89		37.27
Control 6	No malformation	0.47	42.13	43.50	38.17
Average1	NTD	0.03±0.01*	34.95±4.79*	39.67±4.01	39.90±3.45
	Control	0.28±0.09	40.53±2.94	39.70±2.25	36.72±3.79
p		0.00	0.03	0.99	0.16

hypomethylation of Nespas gDMR and folate





summary

- ✓ **Mice embryos developmental dysplasia in response to folate deficiency.**
- ✓ **Parental folate deficiency was associated with loss of imprinting in GNAS gene.**
- ✓ **Parental folate deficiency increased expression levels of Nespas and Exon1A companied with aberrant cAMP signaling.**
- ✓ **NTDs with lower folate concentration had a reduced methylation level of the Nespas gDMR.**


NTD Research Group
THANKS