First Central and Eastern European Summit on pre-conception Health and Prevention of Birth Defects

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Folate and clefts of the lip and palate – a UK based case-control study: Biochemical and genetic analysis

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(on behalf of the ITS MAGIC collaboration, UK)

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Background

In most observational studies, maternal multivitamin intake in early pregnancy is associated with lower risk of orofacial clefts, but data relating specifically to folic acid intake or maternal folate status are inconsistent or unreliable.

Common genetic variation in folate related pathways may be an important factor to consider. Investigating the effect of genetic variation in folate metabolism along with folate intake and blood folate status on relative risk of orofacial clefts:

- May help corroborate other evidence
- Will enable consideration of gene-nutrient interaction.

Background

In the UK Case Control study of mothers of 112 CL(P), 78 CP and 248 controls (Little et al, 2008) there was:

No overall association between CL(P) or CP and dietary or supplemental folic acid intake

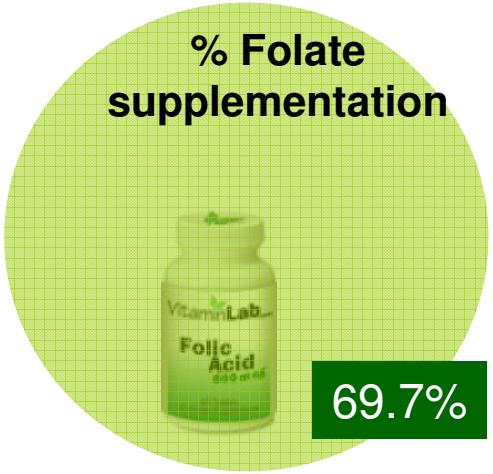
No consistency in the folic acid dose-response effect

Inverse association between both CL(P) and CP and maternal use of multivitamins.

Contrast to some other EU countries eg Netherlands (Van Rooij et al., 2007) and Norway (Wilcox et al., 2007)

Findings from Norwegian study

Norway Population-based case-control study
573 OFC cases, 763 controls, period 1996-2001
Wilcox et al., BMJ 334(7591):464-469, 2007



Summary of findings

Folic acid supplementation (400 ug/day) during early pregnancy reduces CL/P risk by 39%

Lowest risk of CL/P among women with folate rich diets who took folic acid and multivitamins

No protection for CPO

An additional 22% of CL/P cases could be averted if all pregnant women took 400 ug/day of folic acid

MTHFR 677C→T variant

- MTHFR is an key folate enzyme that regulates the distribution of folate co-factors within cells.
- The 677C→T variant is a missense mutation, causing a functional change in the protein that results in reduced activity and an increased dependence on folate and riboflavin compared to the wild-type.
- Between 30 -50% of individuals worldwide have at least one T allele.
- The TT genotype is associated with increased risk of neural tube defects and CVD but may be protective against some cancers under certain nutritional conditions

Effect of the TT genotype on nutritional status

- TT homozygotes have approximately 20% lower folate status than other genotype groups.

- TT homozygotes have higher plasma homocysteine concentrations than expected for a given folate status. This is highly significant at low folate and riboflavin status

- The dietary requirement for both folate and riboflavin may be higher in the 5% to 25% of persons worldwide who are homozygous for this genetic variant

MTHFR 677C→T and OFC

- Several studies found the MTHFR TT genotype to be a maternal risk factor for CL(P)
  - Gaspar et al., 1999; Blanton et al., 2000; Martinelli et al., 2001; Blanton et al., 2002; Sholtersuk et al., 2003; van Rooij et al., 2003; Gaspar et al., 2004; Pezzetti et al., 2004; Mostowska et al., 2006
- One study found an inverse association between CL(P) and presence of the *MTHFR* 677C→T variant in the mother
  - Jugessur et al., 2003
- Most previous studies found no association between infant *MTHFR* genotype and CL(P)
  - Tolarova et al., 1998; Shaw et al., 1998; Gaspar et al., 1999; Mills et al., 1999;

Aims

To determine the associations between non-syndromic cleft lip with or without cleft palate (CL(P)) and cleft palate only (CPO), on biochemical measures of folate status and the *MTHFR* 677C→T variant in an area where the prevalence at birth of neural tube defects has been high and flour is not fortified with folic acid.

Participants for MTHFR analysis

- Affected families (mother, father, case) were recruited from the Scottish mainland and from the Manchester and Merseyside areas of England between 1997 and 2001.
  - 190 cases families (112 CL(P) and 78 CPO)
  - 11 Pierre Robin sequence and 1 micrognathia were included in this total.
  - Other recognised syndromes were excluded
- 248 unaffected control families were recruited from the same areas using the Community Health Index in Scotland and GP practices in England, matched by case post-code.
- Participants were approached at the infants average post-natal age of 5 months (for cases) and 6 months (for controls).
- Mouth-wash or buccal cell samples were collected for genotype analysis.
- A detailed interview and food-frequency questionnaire was administered to mothers.

Participants for maternal folate analysis

- The maternal FFQ at interview included detailed information on total folate intake.
- Mothers participating in the study were asked to additionally provide a blood sample at 12 months.
- At recruitment, 183/190 (96%) of case mothers and 231/248 (93%) of control mothers agreed to give bloods. Of these, 130 (71%) mothers of cases and 125 (50%) mothers of controls, were eligible and available to participate at 12 months.
- Additional information on dietary intake and breast-feeding practices were taken at the time of blood sampling.
- Non-fasting bloods were taken into K-EDTA for plasma folate, red cell folate and plasma homocysteine analysis.

Methods

- MTHFR 677 genotyping was determined using the LightCycler (Roche) real-time PCR system.
- Plasma and red cell folates were analysed by microbiological assay (*Lactobacillus Casei*).
- Plasma homocysteine was measured by reverse phase HPLC using a D30 Hcy Homocysteine Assay Kit.
- All assays were conducted blind to case-control status, or whether the subject was an infant or a parent.

Statistical analysis

- Data were analyzed using STATA software.
- The outcomes were risk of CL(P) and CPO as separate entities.
- The primary analysis was related to the association of these defects with
  (1) Maternal MTHFR genotype, assessed using case-control and case-parent triad analysis
  (2) Maternal red cell folate and plasma homocysteine at 12 months post delivery, assessed by case-control analysis.
- Secondary analyses examined risk associations with case MTHFR genotype, maternal plasma folate and interactions between maternal genotype and folate intake.
- Unconditional logistic regression was used to estimate odds ratios (OR) in case-control analysis, adjusting for sex and season of birth.
- Log-linear modelling was used to analyze case-parent triads and incomplete triads. Adjustments for incomplete data were made using expectation maximisation algorithms in a likelihood framework.
- Plasma homocysteine, red cell and plasma folate were categorized into quartiles.
- Joint effects of maternal genotype and folate intake were tested by comparison of effects above and below the median of residuals of folate intake.
- In all models, odds ratios and relative risks were estimated for TT and CT genotypes using CC as the reference group.

Distribution of MTHFR genotypes in case and control families by cleft type

Type of subject	Number, by *MTHFR* genotype			T allele frequency	HWE		Number	
	CC	CT	TT		D	p	Missing	Total
CL(P)								
Infant	39	47	10	0.35	-0.01	0.45	16	112
Mother	46	42	8	0.30	-0.01	0.71	16	
Father	32	40	14	0.40	0.01	0.80	26	
CPO								
Infant	30	32	7	0.33	-0.01	0.72	9	78
Mother	35	29	7	0.30	0.01	0.78	7	
Father	25	24	7	0.34	0.01	0.74	22	
Control								
Infant	94	101	29	0.35	0.00	0.82	24	248
Mother	86	119	21	0.36	-0.03	0.03	22	
Father	88	91	12	0.30	-0.03	0.07	57	

HWE, Hardy Weinberg equilibrium; D, disequilibrium coefficient; p, p-value associated with chi-square test for departure from HWE

Case-control analysis of oral clefts and maternal *MTHFR* 677C→T genotype

	***MTHFR* Genotype**		
Maternal	**CC**	**CT**	**TT**
Controls	**86**	**119**	**21**
CL(P)	**46**	**42**	**8**
OR* (95% CI)	**1.0 (Ref)**	**0.7 (0.39-1.10)**	**0.7 (0.28-1.71)**
OR† (95% CI)	**1.0 (Ref)**	**0.8 (0.31-1.01)**	**0.6 (0.22-1.58)**
CPO	**35**	**29**	**7**
OR* (95% CI)	**1.0 (Ref)**	**0.6 (0.35-1.12)**	**0.8 (0.30-1.99)**
OR† (95% CI)	**1.0 (Ref)**	**0.6 (0.30-1.11)**	**0.7 (0.24-1.88)**
OFC	**81**	**71**	**15**
OR* (95% CI)	**1.0 (Ref)**	**0.6 (0.42-0.98)**	**0.8 (0.37-1.58)**
OR† (95% CI)	**1.0 (Ref)**	**0.6 (0.35-0.92)**	**0.7 (0.31-1.49)**

*Adjusted for sex and season of birth (matching variables) only
† Also adjusted for infant genotype

Case-control analysis of oral clefts and infant *MTHFR* 677C→T genotype

		***MTHFR* Genotype**		
Infant		**CC**	**CT**	**TT**
Controls		**94**	**101**	**29**
CL(P)		**39**	**47**	**10**
OR*	**(95% CI)**	**1.0 (Ref)**	**1.1 (0.65-1.95)**	**1.0 (0.37-2.09)**
OR†	**(95% CI)**	**1.0 (Ref)**	**1.1 (0.66-1.98)**	**1.0 (0.40-2.31)**
CPO		**30**	**32**	**7**
OR*	**(95% CI)**	**1.0 (Ref)**	**1.1 (0.58-1.97)**	**0.7 (0.28-2.00)**
OR†	**(95% CI)**	**1.0 (Ref)**	**1.0 (0.56-1.94)**	**0.8 (0.28-2.12)**
OFC		**69**	**79**	**17**
OR*	**(95% CI)**	**1.0 (Ref)**	**1.1 (0.68-1.68)**	**0.8 (0.41-1.70)**
OR†	**(95% CI)**	**1.0 (Ref)**	**1.1 (0.67-1.68)**	**0.9 (0.42-1.79)**

•Adjusted for sex and season of birth (matching variables) only
† Also adjusted for maternal genotype

Finding: MTHFR C677T

- Maternal T allele confers protective tendency for both CL(P) and CP……
  ….but not statistically significant

- Little or no effect of infant genotype

Case-parent analysis of association between maternal MTHFR genotype and oral clefts

	Analysis taking into account incomplete trios RR (95% CI)			Analysis restricted to complete trios RR (95% CI)		
	CL(P)	CPO	OFC	CL(P)	CPO	OFC
Triads	68	47	115	68	47	115
Case+1 parent	20	19	39	-	-	-
Case-only	7	2	9			
Maternal MTHFR genotype						
CC (Ref)	1.0	1.0	1.0	1.0	1.0	1.0
CT	0.8 0.46-1.44	0.6 0.26-1.41	0.7 0.46-1.16	0.7 0.34-1.36	0.6 0.24-1.71)	0.7 0.38-1.18
TT	0.4 0.14-1.10	0.8 0.28-2.04	0.5 0.27-1.10	0.4 0.12-1.13	0.5 0.11-1.85	0.4 0.16-0.95

Case-parent analysis of association between case MTHFR genotype and oral clefts

	Analysis taking into account incomplete trios RR (95% CI)			Analysis restricted to complete trios RR (95% CI)		
	CL(P)	CPO	OFC	CL(P)	CPO	OFC
Triads	68	47	115	68	47	115
Case+1 Parent	20	19	39	-	-	-
Case only	7	2	9			
Infant MTHFR genotype						
CC (Ref)	1.0	1.0	1.0	1.0	1.0	1.0
CT	1.3 0.74-2.19	0.9 0.44-1.77	1.1 0.73-1.72	1.6 0.83-3.23	1.0 0.43-2.14	1.3 0.79-2.20
TT	0.8 0.30-2.38	1.1 0.40-3.24	0.9 0.44-1.93	1.3 0.39-4.12	0.7 0.16-2.72	1.0 0.39-2.36

MTHFR genotype: TDT

- Again the maternal T allele seems to have a tendency towards protection (when compared to the CC) for both types of cleft, but all 95% CI's contain unity.

- Lack of association between case MTHFR genotype and oral clefts

Summary of MTHFR results

- Maternal CT and TT genotypes may be protective against CL(P) and CPO in this population, from both case-control and family triad analysis.
- Infant MTHFR 677C→T genotype did not affect risk of OFCs.

Oral clefts and maternal red cell folate one year after birth of index child

	Red Cell Folate			
Quartile	Q1	Q2	Q3	Q4
CL(P) vs. controls				
µg/L §	103.5-323.5	325-444	457-579	584-2228
CL(P)/Controls	30/20	12/38	19/31	17/32
OR* (95%CI)	1.0 (Ref)	0.2 (0.08-0.56)	0.5 (0.21-1.31)	0.5 (0.18-1.18)
CPO vs. controls				
µg/L §	107-355	360-491.5	492.5-605.5	607.5-2228
CPO/Controls	11/33	13/29	9/34	18/25
OR* (95%CI)	1.0 (Ref)	1.6 (0.56-4.38)	0.9 (0.32-2.67)	3.22 (1.14-9.10)
OFC vs. controls				
µg/L §	103.5-334	337.5-466.5	470-599	600-2228
OFC/ Controls	39/24	25/37	30/33	35/27
OR* (95%CI)	1.0 (Ref)	0.4 (0.20-0.93)	0.8 (0.36-1.72)	1.1 (0.52-2.51)

§ Ranges for mothers of cases and controls combined.
* adjusted for sex and season of birth (matching variables), maternal education and child's ethnic group

Findings: Biochemical analysis

- Maternal red cell folate one year after birth of the index child v control mothers…
- In the highest quartile tends to reduce the risk for a child with CL(P)
- In the highest quartile tends to elevate the risk for a child with CP

Oral clefts and maternal serum homocysteine levels infants one year after birth of index child

	Serum Homocysteine			
Quartile	Q1	Q2	Q3	Q4
CL(P) vs. controls				
µmol/L §	3-7	7.1-8.7	8.8-11	11.3-27
CL(P)/Controls	18/33	13/33	19/29	27/21
OR* (95%CI)	1.0 (Ref)	0.6 (0.22-1.55)	1.1 (0.46-2.74)	1.6 (0.63-4.30)
CPO vs. controls				
µmol/L §	3-7	7.1-9.2	9.3-12	12.1-27
CPO/Controls	10/34	13/33	14/26	13/30
OR* (95%CI)	1.0 (Ref)	1.6 (0.59-4.53)	2.2 (0.81-6.18)	1.6 (0.58-4.33)
OFC vs. controls				
µmol/L §	3-6.8	6.9-8.7	8.8-11.4	11.5-27
OFC/ Controls	30/32	25/34	33/30	38/20
OR* (95%CI)	1.0 (Ref)	0.7 (0.32-1.47)	1.1 (0.50-2.30)	1.5 (0.67-3.58)

Findings: Biochemical analysis

- Maternal homocysteine one year after birth of the index child v control mothers…

- No effect for either CL(P) or CP

Case-control analysis of interaction between maternal *MTHFR* genotype and total folate intake

Total folate Intake	Maternal *MTHFR* Genotype Cases/controls OR (95% CI)		
	CC	CT	TT
CL(P)			
Below median	24/37 1.0 (Ref)	28/52 0.78 (0.38-1.60)	7/11 0.86 (0.28-2.62)
Above median	22/49 0.87 (0.41-1.85)	14/67 0.37 (0.17-0.83)	1/10 0.17 (0.02-1.52)
	p interaction = 0.32		
CPO			
Below median	19/37 1.0 (Ref)	17/52 0.72 (0.32-1.63)	5/11 0.80 (0.23-2.82)
Above median	16/49 0.85 (0.37-1.96)	12/67 0.41 (0.17-0.97)	2/10 0.42 (0.08-2.23)
	p interaction = 0.72		
OFC			
Below median	43/37 1.0 (Ref)	45/52 0.71 (0.39-1.33)	12/11 0.84 (0.32-2.19)
Above median	38/49 0.84 (0.44-1.58)	26/67 0.38 (0.20-0.74)	3/10 0.28 (0.07-1.15)
	p interaction = 0.43		

Case-parent triad analysis*

Total Folate Intake →	**Below Median**	**Above Median**
	RR (95% CI)	RR (95% CI)
CL(P) – 95 families	48	47
Maternal MTHFR genotype		
CC (Ref)	1.0	1.0
CT	1.1 (0.51-2.44)	0.5 (0.25-1.19)
TT	1.0 (0.21-4.42)	0.2 (0.03-0.76)
CPO –68 families	34	34
Maternal MTHFR genotype		
CC (Ref)	1.0	1.0
CT	0.7 (0.25-1.96)	0.4 (0.08-1.60)
TT	1.3 (0.34-5.20)	0.3 (0.06-1.73)
OFC –163 families	82	81
Maternal MTHFR genotype		
CC (Ref)	1.0	1.0
CT	0.9 (0.47-1.67)	0.5 (0.22-1.00)
TT	1.4 (0.53-3.95)	0.2 (0.04-0.53)

***Including incomplete trios and trios when cases had other anomaly**

MTHFR / Folate interaction

- For C677T polymorphisms, the T allele has a protective tendency, but only in those mothers whose total folate intake is above median.

Conclusions: UK study

- Higher levels of RBC and serum folate were associated with a decreased risk for CL(P)
- By contrast, higher levels of RBC and serum folate showed a trend towards increased risk for CPO.
- Maternal homozygosity and heterozygosity for the *MTHFR* 677C→T genotype were associated with a reduced risk of both CL(P) and CPO.
- This effect was confined to mothers whose intake of total folate was above the median.

Eurocran study

- European Collaboration on Craniofacial Anomalies
- Funded by European Commission FP5 programme (2001-2005)
- Involved countries from Western, Central and Eastern Europe
- 5 Work Packages on OFC: Treatment, GEI, genetics, chromosomes and website.


8th European Craniofacial Congress
Bilbao June 20-23 2007
EUROCRAN
EUROPEAN COLLABORATION ON
CRANIOFACIAL ANOMALIES
WP2
Michele Rubini, Julian Little, Regine Steegers-Theunissen,
Borut Peterlin, Juhn Scott, Anne Molloy, Peter Mossey

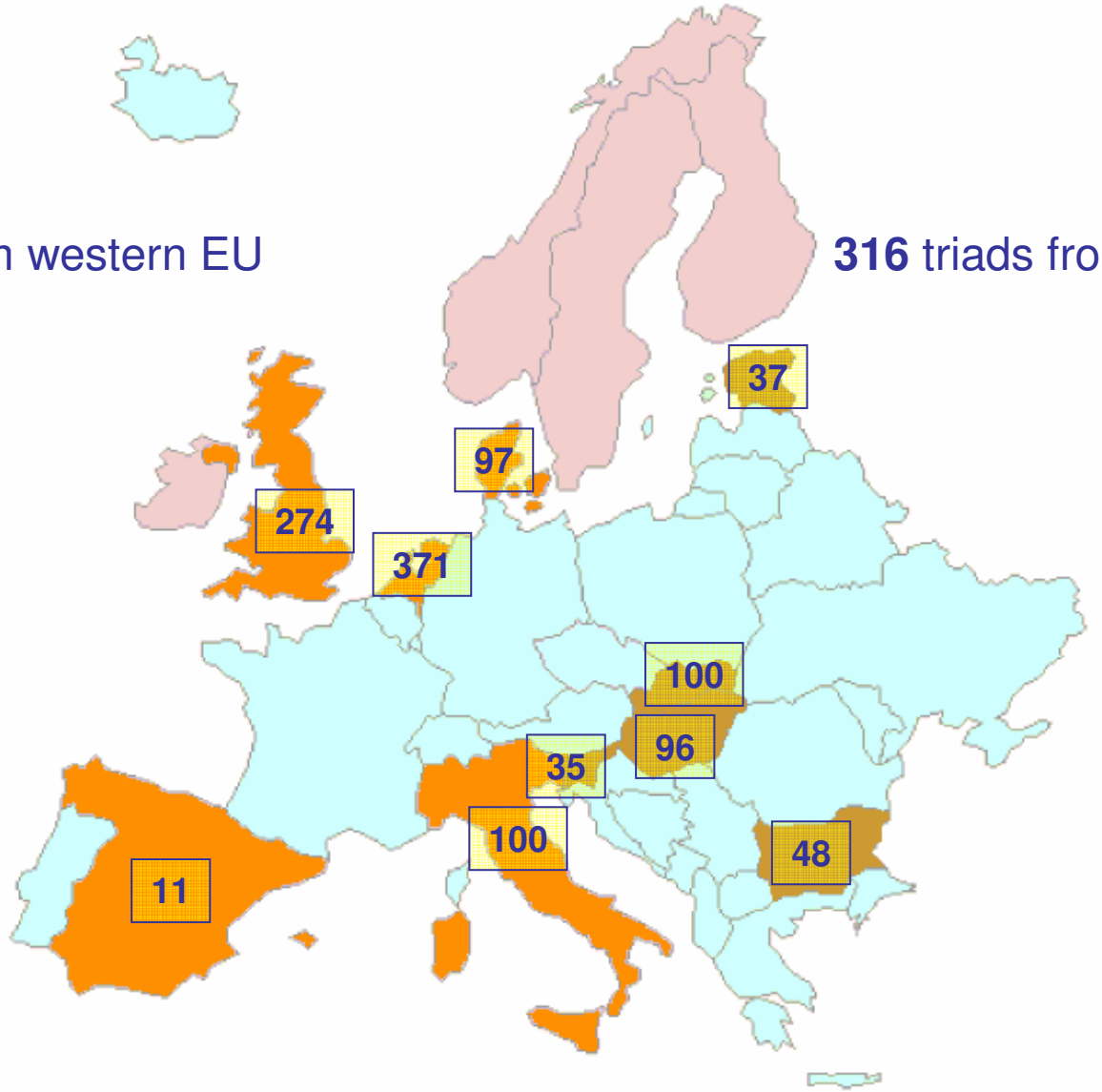
WP2- Triads recruitment from 10 EU countries

1169 triads between 2001 and 2005

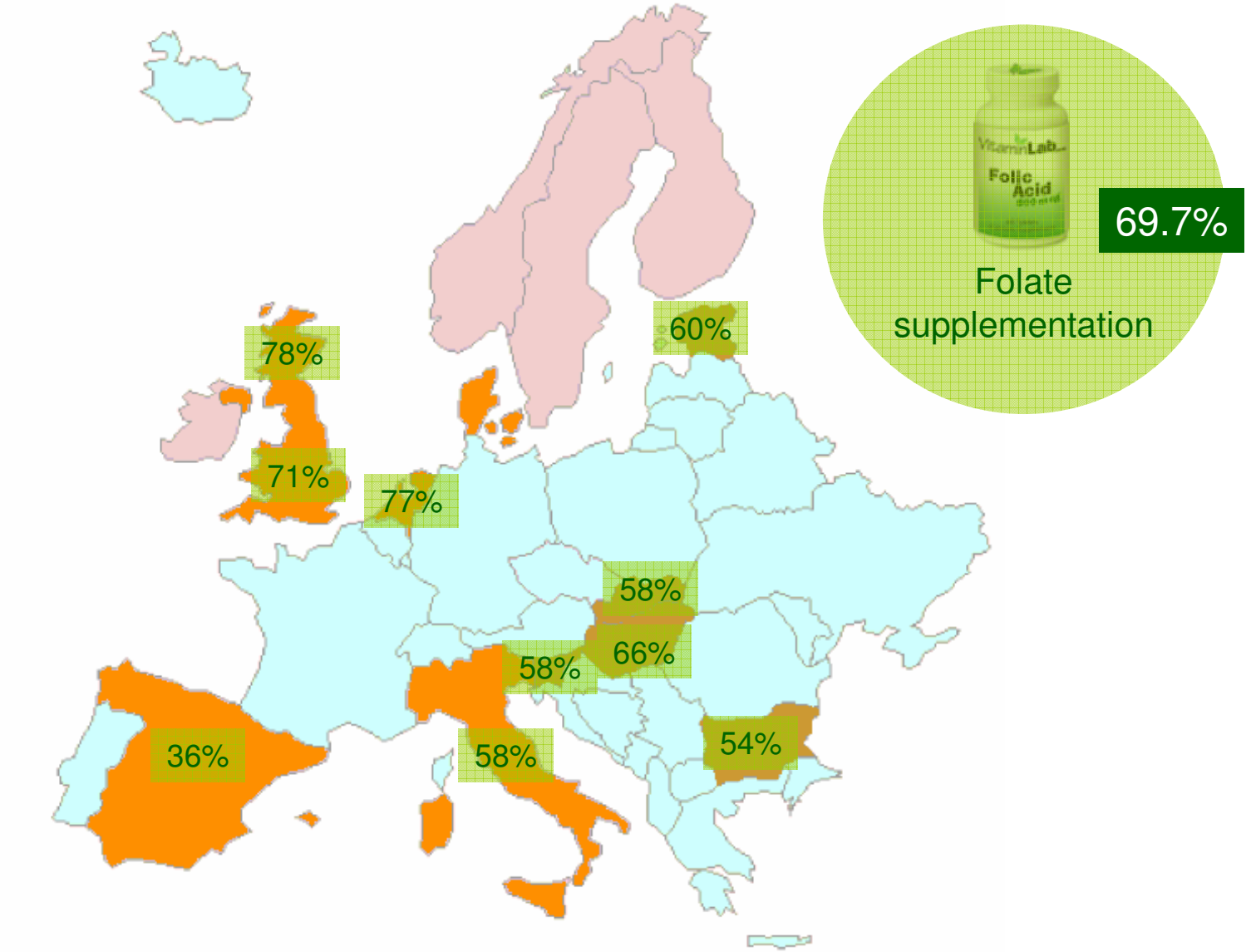
81.5% of target

853 triads from western EU

316 triads from eastern EU



Supplementation with folic acid during pregnancy



EUROCRAN SUMMARY

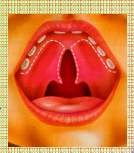
Slight protective effect of 677TT genotype in mothers, evident only in FA supplemented mothers (significant for CL/P)

MTHFR 677TT

CL/P

MTHFR 677TT

MTHFD1 1958AA



CPO

Folic Acid Supplementation in early pregnancy

Significant reduction of CPO risk in 1958AA mothers supplemented with FA (opposite effect of FA for CL/P)

Significant reduction in the risk of OFC in MTHFR 677TT children

FA supplementation does not interact with child MTHFR or MTHFD1 genotype

Overall conclusions

Still no consistency in evidence for:

- Protective effect of maternal dietary and / or supplemental folic acid
- Higher dose folic acid conferring greater protection
- Maternal or infant MTHFR polymorphisms conferring increased risk
- Gene-environment interaction related to folate metabolism / intake
- Elevated homocysteine conferring increased risk

Overall Conclusions

There is some consistency of evidence for:

- Maternal multivitamin use conferring protection (both CP / CL(P)
- Lower risk with maternal C677T genotype (and greater in supplemented mothers)

Overall Conclusions

- There is extreme heterogeneity between studies for:
- Dietary folate, folic acid supplements, fortification, biochemical measures and gene polymorphisms in the folate pathway.
- Therefore single centre studies are of little use on their own.

Future studies on OFC aetiology

- Must be multi-centre and strive for very large numbers
- Should be collaborative, or at least collect the same core dataset.
- Examine both genetic and environmental factors
- Use genome wide and candidate gene approaches
- RCT would help to clarify the dose response issue for OFC

Congratulations

To the organisers of :

First Central and Eastern European Summit on pre-conception Health and Prevention of Birth Defects

This is an important initiative in the field of birth defects research with global implications and complements other initiatives in the field.

William Christie Shaw – University of Manchester, UK
Gunvor Semb – University Hospita Rikshospitalet, Sweden
Peter Mossey – University of Dundee, UK
Mike Dixon – University of Manchester, UK
Regine Steegers-Theunissen – Rotterdam, Netherlands
Roberto Brusati, **Giovanna Garattini** – Ospedale San Paolo, Milan, Italy
Gonzalo Gonzàles-Landa – Hospital de Cruces, Barakaldo Vizcaya, Spain
Anne Marie Kuijper-Jagtman – University of Nijmegen, Netherlands
Annette Lohmander-Agerskov – Goteborg University, Sweden
Kare Molsted - Speech Hearing Inst., Hellerup, Danmark
Jorma Rautio – University of Helsinki, Finland
David FitzPatrick – University of Edinburg, UK
Michele Rubini - University of Ferrara, Italy
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