FOLATE RELATED RISK FACTORS AND OROFACIAL CLEFTING IN HUMAN

EPIDEMIOLOGICAL AND BIOLOGICAL STUDIES

FOLIUMZUUR GERELATEERDE RISICOFACTOREN EN OROFACIALE SCHISIS IN DE MENS

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Thesis, Erasmus University Rotterdam, The Netherlands

The research in this thesis has been performed at the Department of Obstetrics and Gynaecology, division Obstetrics and Prenatal Medicine and the Department of Clinical Genetics of the ErasmusMC University Medical Centre, Rotterdam, The Netherlands.

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Paranimfen:	Michael Aletrino Sander Bodmer

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PART ONE

GENERAL INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

Cleft lip with or without palate

Definition and Epidemiology

Orofacial clefting (OFC) is a group of congenital malformations characterized by closure defects of the lip, jaw and/or palate. In general, three subgroups of OFC are distinguished, e.g., isolated clefts of the lip or the palate and clefts of the lip and the palate. Based on embryology, the isolated cleft of the lip and cleft lip together with cleft palate are considered a continuum of the same malformation, which will be referred to as cleft lip with or without palate (CL/P). This is in contrast to the isolated cleft palate (CP) which is regarded to be etiologically different. With a live birth prevalence rate of 11.8 per 10,000, CL/P is the third most frequent congenital malformation in The Netherlands [1]. This is more than twice as high as the birth prevalence rate of CP of 2.4 per 10,000 live births. Figure 1.1 shows the birth prevalence rates of CL/P and CP in the period from 1999 to 2008. Around 25% of the CL/P and 40% of the CP cases occur in combination with other malformations or as part of a syndrome and are considered syndromic clefts. In the studies presented in this thesis we focused on non-syndromic CL/P and CP that were not associated with other major congenital malformations.

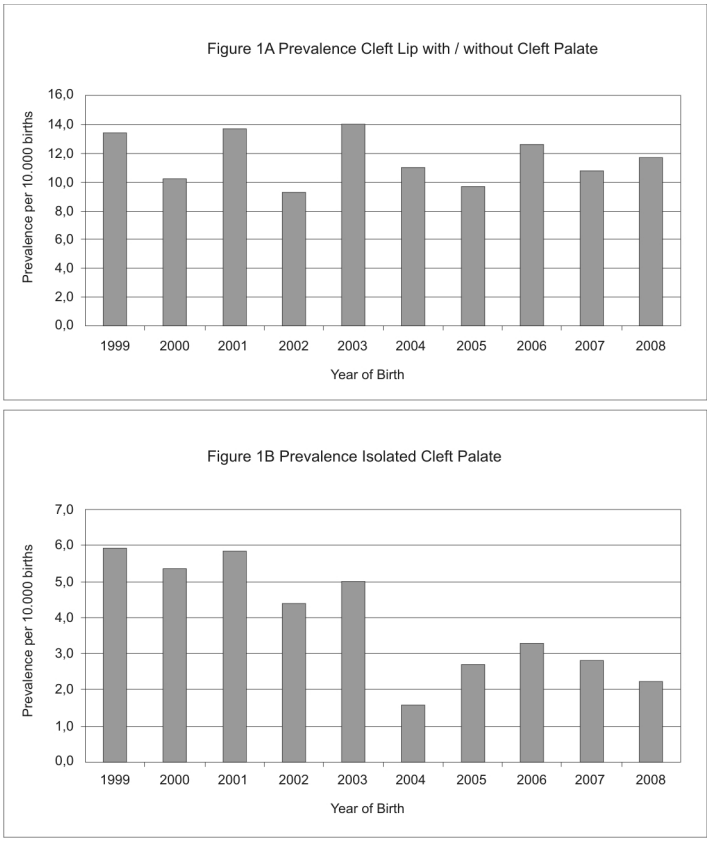
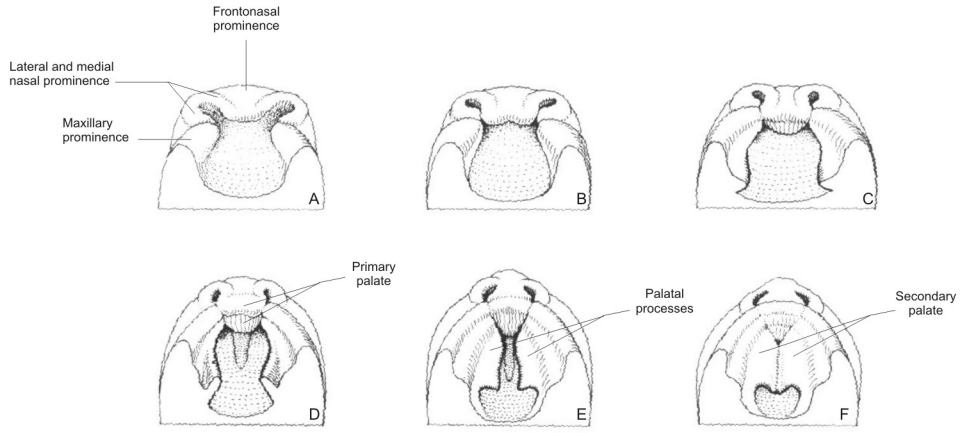


Figure 1 Prevalence of orofacial clefting

A,B Birth prevalence rates (per 10,000 live births) of CL/P and CP, respectively, from 1999-2008 in the Dutch provinces Groningen, Friesland and Drenthe. Source: EUROCAT Northern Netherlands

Figure 2 Schematic overview of palatal development



Adapted from: Klinische anatomie en embryologie (1997), Bunge publishers

Embryology – primary and secondary palate

From the fifth week of embryonic development onwards the primitive oral cavity is formed (figure 2a). The cavity is surrounded by five prominences, from which the nose, lip and palate arise. Firstly, two outcurvings are formed from the frontonasal prominence, which connect at their lateral side with the lateral nasal prominence and the maxillary prominence (figure 2b). These three prominences form the primary nasal cavity. This is followed by the merging of two medial nasal prominences, which define the middle sections of the lip and the jaw from which the upper four teeth will grow out and the first part of the palate, together referred to as primary palate (figure 2c,d). The partial or total absence of the fusion of these tissues causes clefting of the lip with possible additional clefting of the jaw and palate.

From the seventh week after conception two palatal processes develop from the maxillary prominences and grow into the oral cavity in the medial direction (figure 2d,e). When these processes reach each other in the middle they merge, starting from the primary palate to the back of the oral cavity, and form the secondary palate (figure 2f). Failure of the merging of the secondary palate can be caused by an already present cleft in the primary palate or can occur separately, with a normal developed primary palate.

Etiology

The etiology of OFC is heterogeneous, in which interaction between subtle genetic mutations and environmental exposures are implicated. Table 1 shows an overview of genes and loci that are reported to play a role in the development of the lip and palate and have been associated with OFC.

Table 1 Genes and loci associated with non-syndromic orofacial clefting.

Loci	Gene	Reference
1p22.1	ABCA4	[2]
1p21-31		[3]
1q32.3-q41	IRF6	[4-13]
2p13	TGFα	[13-32]
2q32-33	SATB2	[33]
2q33	SUMO1	[34-37]
3q27-28	TP73L	[13, 38-47]
4p16		[48]
4q21-q31		[3, 49-52]
4q16	MSX1	[53-63]
5p13-p12	FGF10	[64-65]
6p24-p23		[66-78]
8p11.2-p11.1	FGFR1	[64-65, 79]
8q24.21		[2, 11, 80-83]
9q21-33	FOXE1	[13, 84]
10q26	FGFR2	[64-65, 85]
10q25.3	KIAA1598, VAX1	[11, 86-87]
11q23.3	PVRL1	[88-93]
12q13.11	COL2A1	[94]
13q33.1-q34		[95-96]
14q24	TGFβ	[33, 54, 97-103]
14q21-q23	BMP4	[13, 104-106]
17q21.1	RARα	[17, 30, 77, 100, 107-111]
17q22	NOG	[86]
19q13	PRR2	[30, 112-116]
20q12	MAFB	[2]
22q12.3	MYH9	[117]

Adapted from Carinci et al. [118]

From the early eighties it became more clear that the development of the embryo is not only determined by genes, but is also subject to environmental influences, such as periconception exposure to medication, tobacco smoke, alcohol and folic acid. Such lifestyle determinants most likely coincide with factors, such as maternal age, parental education and socio-economic status, which also affect CL/P risk in the offspring (see also table 2). Periconceptional intake of folic acid tablets, but also sufficient intake of natural food folates have been found to reduce the CL/P risk up to 50% [125-127, 129-130, 151], though the results are sometimes ambiguous [152-153]. Folic acid supplement use also shows a risk reduction for other congenital malformations, such as neural tube defects and congenital heart defects [125, 154-156].
The mechanisms by which folic acid supplement use protects against CL/P is still not clarified. Despite the lack of this knowledge and the impact of folate on gene and protein interactions this has resulted in mandatory folic acid food fortification in several countries [157] with possible detrimental side effects such as increased colon cancer [158].

Folate

The function of folate (figure 3) comprises of the synthesis of one-carbon groups, which are necessary for the synthesis of amino acids, such as choline, serine, cysteine and glycine, the purines adenine and guanine, the pyrimidine thymine and

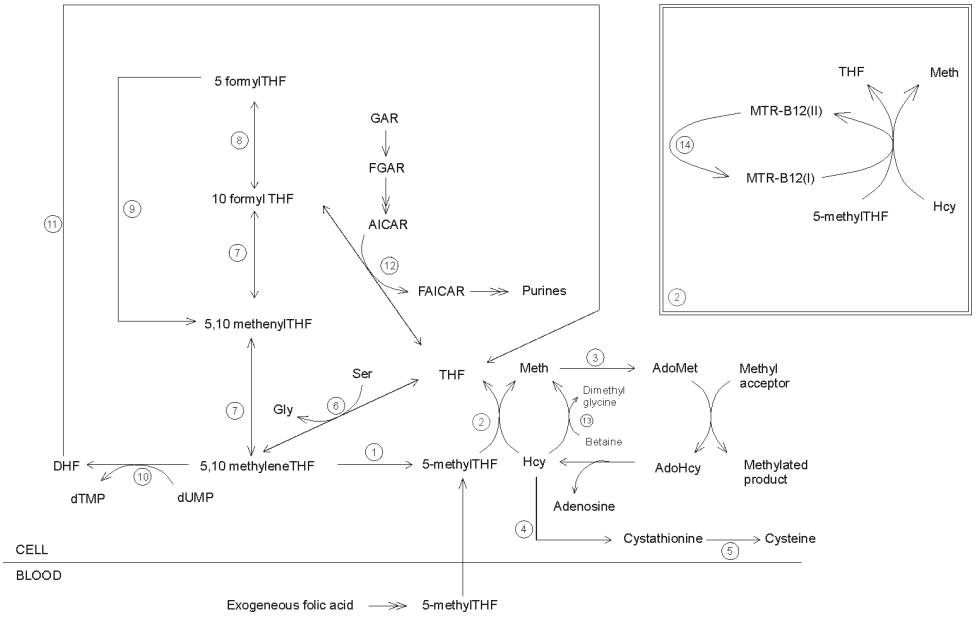
Table 2 Environmental determinants associated with the risk of orofacial clefting in the offspring

Maternal characteristic or environmental determinant	Interacting gene	Author, Year	Reference
Maternal age		Kallen 1996, DeRoo 2006, Bille 2005	[119-121]
Educational level		Krapels 2006	[122]
Socio-economic status		Clark 2003, Carmichael 2003	[123-124]
Folic acid supplement use		Tolarova 1982, Czeizel 1999, Itikala 2001, Shaw 2002, Botto 2004, Wilcox 2007	[125-130]
	MTHFR	Shaw 1998, Van Rooij 2003, Mills 2008, Chevrier 2007	[131-134]
	TGFα	Shaw 1998	[135]
	IRF6	Wu 2010	[136]
Medication		Carmichael 1999, Hernandez-Diaz 2000	[137-138]
	NAT-2	van Rooij 2002	[139]
	MDR-1	Bliek 2006	[140]
Smoking		Little 2004, Honein 2007	[141-142]
	NAT-2	Van Rooij 2002, Shi 2007	[139, 143]
	MSX1	Van den Boogaard 2008	[144]
	GST	Lammer 2005, Shi 2007	[143, 145]
Alcohol		Romitti 2007	[146]
	ADH1C	Shaw 1999, Chevrier 2005, Boyles 2010	[147-149]
Myo-inositol, glucose and zinc		Krapels 2004	[150]

the remethylation of homocysteine into methionine. The methionine derivative S-adenosylmethionine is the main methyl group donor for the methylation of DNA, RNA, proteins and lipids [159].
Antioxidant properties of folate have also been reported [160]. Several *in vitro* studies with human cell lines have shown that folate deficiency increases apoptosis [161], uracil misincorporation [162], causes DNA and protein hypomethylation [163-165], DNA strand breakage [162, 166] and chromosome aneuploidy [167-168]. However, these results cannot explain the strong protective effects of folic acid supplement use during pregnancy.
The interaction of folate on a molecular level might be best understood and targeted with the methylation hypothesis, indicating a role for folate in epigenetic regulation and post-transcriptional modification [169]. Because methyl-groups are derived from the diet, in particular of folate as precursor of S-adenosylmethionine, folate is considered a modifier of gene expression [170]. Reports on the influence of folate on DNA methylation [164-165, 171], gene-expression [172] and DNA hypomethylation resulting from hyperhomocysteinemia in DNA of lymphocytes [173] support this hypothesis. This is in line with evidence that the maternal nutritional state alters the epigenetics of the fetal genome [174-175]. From this point of view, folate and/or its derivatives might play a role in the programming and expression of (developmental) genes, implicated in embryonic growth and development. A folate deficiency may then cause abnormal tissue specific gene expression patterns resulting in congenital malformations such as OFC. A previous study by Spiegelstein et al [176] using a murine Folbp1 knockout model showed folate responsiveness of several genes including transcription factors, G-proteins, growth factors, methyltransferases and cell proliferation related genes. A second

study by Courtemanche et al [166] showed expression of genes involved in DNA repair, mitochondrial and folate metabolism. These limited results, however, need further exploration in human using genome wide scans to further understand the role of folate in CL/P development.

Figure 3 Folate metabolism



THF= tetrahydrofolate, DHF= dihydrofolate, Meth= methionine, Hcy= homocysteine, AdoMet= S-adenosylmethionine, AdoHcy= S-adenosylhomocysteine, (F)GAR= (formyl) glycinamide ribonucleotide, (F)AICAR= (formyl) aminoimidazole carboxamide ribonucleotide, Ser= serine, Gly= glycine, 1= 5, 10-methylenetetrahydrofolate reductase (MTHFR), 2= methioninesynthase (MS), 3= methionine adenosyltransferase (MAT), 4= cystathionine β-synthase (CBS), 5= γ-cystathionase (GC), 6= serine hydroxymethyltransferase (SHMT), 7= 5, 10-methylenetetrahydrofolate dehydrogenase (MTHFD), 8= equilibrium (non-enzymatic), 9= 5-formyltetrahydrofolate cyclodehydrase (FTHFD), 10= thymidylate synthase (TS), 11= dihydrofolate reductase (DHFR), 12= aminoimidazole carboxamide ribonucleotide formyltransferase (AICRFT), 13= betaine-homocysteine methyltransferase (BHMT), 14= methionine synthase reductase (MTRR)

Objectives of the Thesis

From this background it has become clear that folic acid supplement use plays an important role in the development of non-syndromic OFC though the underlying mechanisms are not yet clarified. In order to gain more insights in the role of environmental exposures and underlying mechanisms, the aims of this thesis are to identify:

Part 1 Epidemiological Studies: new risk factors for CL/P and the relationship with folic acid supplement use

Part 2 Biological Studies: the effects of natural folate supplement use on a cellular, proteomic and genetic level

Outline of the Thesis

In part 1 some new risk factors for CL/P are identified and their relation with folic acid supplement use is determined. Chapter 2 presents a meta-analysis reviewing hyperhomocysteinemia and two functional polymorphisms of MTHFR, an important enzyme in folate metabolism, and the risk of CL/P. Chapter 3 presents a case-control study concerning the effects of periconception medication and folic acid use and carriership of a polymorphism in the detoxification gene ABCB1 on the risk for CL/P. Chapter 4 evaluates the association of the presence of folate receptor blocking antiserum in the maternal blood and the risk for CL/P offspring.
In the second part of this thesis cellular, proteomic and genomic effects have been investigated after natural folate depletion and addition in B-lymphoblasts from children with and without CL/P. Chapter 5 investigates folate responsive gene expression profiles in CL/P affected children using genome-wide expression arrays. In chapter 6, a pilot study is presented in which mass spectrometry peptide fingerprinting of B-lymphoblasts from CL/P and healthy children is used to identify folate responsive proteins. Chapter 7 is an extension of the study described in chapter 6 with larger study groups and more accurate mass spectrometry and software analysis. In Chapter 8 the effects of folate deficiency on chromosome 17 and 21 aneusomy are investigated in CL/P and healthy children.
Finally, in chapter 9 we elaborate on the results and implications of the findings of this thesis.

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PART TWO

EPIDEMIOLOGICAL STUDIES

CHAPTER 2

HYPERHOMOCYSTEINEMIA AND MTHFR POLYMORPHISMS IN ASSOCIATION WITH OROFACIAL CLEFTS AND CONGENITAL HEART DEFECTS. A META-ANALYSIS

Anneke Verkleij-Hagoort, **Bart JB Bliek,** Fakhredin Sayed-Tabatabaei, Nicolette Ursem, Eric AP Steegers, Régine PM Steegers-Theunissen
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Abstract

Several studies have reported an association between hyperhomocysteinemia, 5,10-methylenetetrahydrofolate reductase (MTHFR) polymorphisms and cleft lip with or without cleft palate (CLP), and congenital heart defects (CHDs). However, findings have been inconsistent. A metaanalysis was performed of published studies until September 2006 investigating these associations in both mothers and children. Homocysteine data were provided in two CLP and three CHD studies, and MTHFR polymorphisms were reported in ten CLP and eight CHD studies. Data were analyzed using the random effects model in the Cochrane Review Manager. The pooled odds ratio (OR) of maternal hyperhomocysteinemia was 2.3 (95% CI 0.4–11.9) for CLP, and 4.4 (2.6–7.3) for CHDs. The MTHFR C677T polymorphism and CLP showed pooled ORs of 1.2 (0.9–1.5) in mothers and 1.0 (0.9–1.2) in children, whereas these estimates for the A1298C polymorphism were 1.0 (0.7–1.2) in mothers and 0.9 (0.6–1.2) in children. The MTHFR C677T polymorphism in CHD studies demonstrated a pooled OR of 1.0 (0.8–1.3) for mothers and 1.1 (0.9–1.5) for children. Two studies investigating the maternal A1298C polymorphism in CHDs demonstrated a pooled OR of 1.2 (0.8–1.8). Only one CHD study reported an OR of 1.3 (0.8–2.1) for this polymorphism in children. In conclusion, this meta-analysis demonstrates that maternal hyperhomocysteinemia is a risk factor for CHDs. The MTHFR polymorphisms C677T and A1298C in both mothers and children are not independently associated with CLP or CHDs. Future studies should be performed to investigate the interactions between maternal hyperhomocysteinemia, B-vitamin intake, related polymorphisms and the risk of CLP and CHDs.

Introduction

Orofacial clefting (OFC) and congenital heart defects (CHDs) develop during the first weeks after conception. These defects are common congenital anomalies of multifactorial origin influenced by both genetic and environmental factors [1-2]. Various epidemiologic studies have shown the protective effect of maternal use of multivitamins in the periconceptional period on the risk of having a child with OFC [3], and a child with a CHD [4-6]. However, it is unknown which ingredient(s) in multivitamins are responsible for this risk reduction. Indirect evidence that folic acid is a key factor in orofacial and cardiovascular development has been suggested by a study of Hernandez-Diaz et al. (2000), in which folic acid antagonists were shown to increase the risk of a child with OFC or a CHD. Mothers who used multivitamins containing folic acid in addition to dihydrofolate reductase inhibitors, showed a fivefold lower risk of having a child with OFC or a CHD compared with mothers who did not concomitantly use multivitamins [7]. Folate contributes to the transfer of one-carbon groups as part of nucleotide synthesis, the remethylation of homocysteine to methionine, and the subsequent methylation of DNA, proteins, and phospholipids. Hyperhomocysteinemia and DNA hypomethylation contribute to the development of complex congenital disorders [8]. Therefore, an optimal maternal and embryonic folate status is important for normal embryogenesis. The enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) is an important enzyme in the homocysteine metabolism and catalyzes the conversion of 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate, the predominant

circulating form of folate. The MTHFR gene has at least two functional polymorphisms, C677T and A1298C. The MTHFR 677T allele is associated with reduced enzyme activity, decreased concentrations of folate in serum, plasma, and red blood cells, and mildly increased plasma total homocysteine (tHcy) concentrations [9-10]. The second polymorphism, MTHFR A1298C, also affects MTHFR activity but without biochemical changes [11]. Normal MTHFR activity is crucial to maintain the pool of circulating folate and methionine and to prevent the accumulation of homocysteine [9]. Homocysteine can be considered as a useful and important metabolic marker of the overall folate status. Several studies have reported inconsistent findings on associations between hyperhomocysteinemia, MTHFR polymorphisms, and both OFC and CHD risk. Therefore, we performed a meta-analysis of all published studies until September 2006 investigating, in mothers and children, the associations between hyperhomocysteinemia, MTHFR C677T and A1298C polymorphisms, and the risk of both OFC and CHDs.

Materials and Methods

Studies

Potential relevant studies were identified by using MESH terms and text words in a search of PubMed at the National Library of Medicine, Web of Science, Cochrane library, Scopus and the Genetic Association Database through September 1, 2006. The main search terms were 'cleft lip,' 'cleft palate,' 'heart defects, congenital,' 'homocysteine,' 'methylenetetrahydrofolate reductase (nadph2)' and 'MTHFR.' We also conducted searches on congenital anomalies and malformations in general, because OFC and CHDs may not be specified when a study is related to several congenital malformations. Furthermore, we performed manual searches of reference lists in articles found during the electronic searches. If studies presented overlapping data, only the study with the largest number was included. All studies were published in German or English language. Authors were contacted by email asking them to provide data if the content of the paper was insufficient. This meta-analysis is limited to casecontrol and cohort studies that include data of homocysteine concentrations and/or the MTHFR polymorphisms. It does not include animal studies and studies of case series. Genetics and embryology suggest that clefts of the primary palate that involve the lip and/or palate are different in etiology from clefts that affect the secondary palate and are, therefore, developmentally distinct entities [12-13]. Moreover, patients with cleft lip with or without cleft palate (CLP) represent the largest and most homogeneous group of oral clefts. Therefore, we included only those studies that investigated CLP and excluded the studies concerning isolated cleft palate. In total, we identified two CLP and five CHD studies investigating tHcy concentrations, and 23 CLP and 11 CHD studies that reported on MTHFR polymorphisms. We excluded two CHD studies on tHcy concentrations, because one study population [14] was part of a larger included study [15] and the other study used a different cutoff level for the tHcy concentrations and included older participants as well [16]. Twelve studies on CLP and MTHFR polymorphisms were excluded, because seven studies used a family-based design [17-23], two studies were part of a larger included study [24-25], and three studies only reported allele frequencies [26-27] or incomplete genotype frequencies [28]. In respect of MTHFR polymorphisms in CHD studies,

we excluded three studies that included only cases [29-31]. Therefore, the meta-analysis was performed on tHcy data provided in two CLP [32-33] and three CHD studies [15, 34-35] and on MTHFR polymorphisms reported in ten CLP [36-45] and eight CHD studies [43, 46-52].

Statistical Analysis

Hardy–Weinberg equilibrium (HWE) was examined in all studies that included MTHFR genotype frequencies. The available tHcy and polymorphism data for the meta-analysis were entered in Review Manager (RevMan [Computer Program], version 4.2 for Windows. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2003) and analyzed with RevMan Analyses (version 1.0 for Windows). This program uses the method of moments to calculate the odds ratio (OR) and 95% confidence interval (CI) for the pooled data. The genetic dominant model was used for comparisons of the polymorphism data. Heterogeneity was assessed using the Q test [53]. Nevertheless, we used the random effects model in all analyses. Funnel plots were used to investigate publication bias.

Figure 1 Association between maternal hyperhomocysteinemia and the risk of cleft lip with or without cleft palate and congenital heart defects.

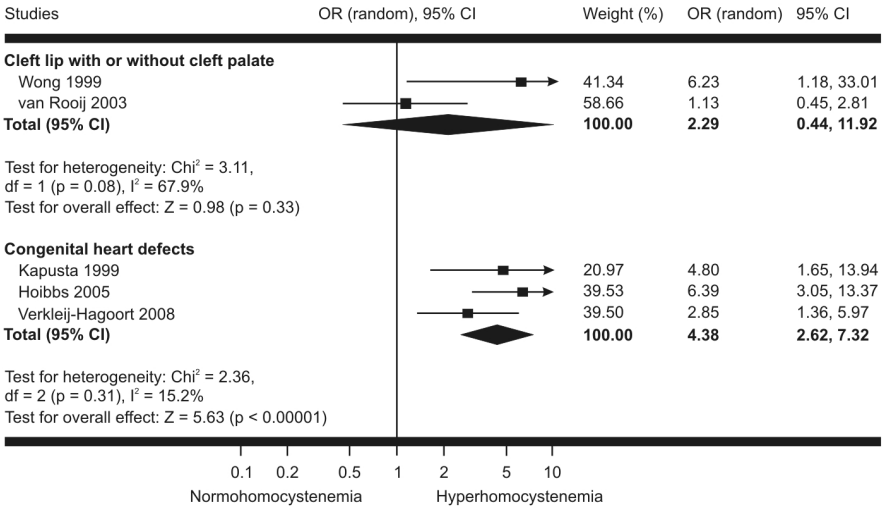


Table 1 MTHFR Genotype Frequencies in CLP Studies

References		Frequency in mothers, n (%)			Frequency in children, n (%)		
C377T polymorphism	**Subjects**	**CC**	**CT**	**TT**	**CC**	**CT**	**TT**
Shaw et al. [1998]	Case	NA	NA	NA	143 (46.1)	127 (41.0)	40 (12.9)
California, USA	Control	NA	NA	NA	156 (40.7)	178 (46.5)	49 (12.8)
Tolarova et al. [1998]	Case	39 (41.9)	37 (39.8)	17 (18.3)	43 (38.8)	49 (44.1)	19 (17.1)
Argentina	Control	39 (46.4)	33 (39.3)	12 (14.3)	46 (43.4)	52 (49.1)	8 (7.5)
Martinelli et al. [2001]	Case	14 (22.2)	36 (57.2)	13 (20.6)	22 (34.4)	30 (46.9)	12 (18.7)
Italy	Control	46 (43.4)	43 (40.6)	17 (16.0)	46 (43.4)	43 (40.6)	17 (16.0)
Grunert et al. [2002]	Case	NA	NA	NA	34 (51.5)	26 (39.4)	6 (9.1)
Germany	Control	NA	NA	NA	90 (48.9)	69 (37.5)	25 (13.6)
Shotelersuk et al. [2003]	Case	46 (68.7)	19 (28.3)	2 (3.0)	84 (77.1)	25 (22.9)	0 (0.0)
Thailand	Control	154 (76.2)	46 (22.8)	2 (1.0)	154 (76.2)	46 (22.8)	2 (1.0)
van Rooij et al. [2003b]	Case	78 (52.7)	55 (37.2)	15 (10.1)	54 (51.4)	45 (42.9)	6 (5.7)
The Netherlands	Control	84 (49.4)	74 (43.5)	12 (7.1)	70 (54.7)	54 (42.2)	4 (3.1)
Gaspar et al. [2004]	Case	174 (51.8)	131 (39.0)	31 (9.2)	327 (50.8)	269 (41.8)	48 (7.4)
Brazil	Control	213 (50.2)	172 (40.6)	39 (9.2)	213 (50.2)	172 (40.6)	39 (9.2)
Nurk et al. [2004]	Case	12 (54.5)	8 (36.4)	2 (9.1)	NA	NA	NA
Norway Control	Control	7,153 (49.5)	6,029 (41.7)	1,280 (8.8)	NA	NA	NA
Pezzetti et al. [2004]	Case	27 (26.0)	47 (45.2)	30 (28.8)	28 (25.5)	58 (52.7)	24 (21.8)
Italy	Control	95 (32.9)	151 (52.2)	43 (14.9)	95 (32.9)	151 (52.2)	43 (14.9)
Mostowska et al. [2006]	Case	60 (49.6)	46 (38.0)	15 (12.4)	NA	NA	NA
Poland	Control	42 (51.9)	33 (40.7)	6 (7.4)	NA	NA	NA
A1298C polymorphism		**AA**	**AC**	**CC**	**AA**	**AC**	**CC**
Tolarova et al. [1998]	Case	56 (65.1)	27 (31.4)	3 (3.5)	67 (62.0)	39 (36.1)	2 (1.9)
Argentina	Control	50 (64.1)	25 (32.0)	3 (3.9)	63 (61.2)	33 (32.0)	7 (6.8)
Grunert et al. [2002]	Case	NA	NA	NA	28 (43.1)	30 (46.1)	7 (10.8)
Germany	Control	NA	NA	NA	77 (41.9)	80 (43.5)	27 (14.6)
Shotelersuk et al. [2003]	Case	30 (44.8)	33 (49.2)	4 (6.0)	55 (50.5)	48 (44.0)	6 (5.5)
Thailand	Control	108 (53.5)	80 (39.6)	14 (6.9)	108 (53.5)	80 (39.6)	14 (6.9)
van Rooij et al. [2003b]	Case	57 (45.6)	52 (41.6)	16 (12.8)	48 (51.0)	34 (36.2)	12 (12.8)
The Netherlands	Control	76 (47.8)	67 (42.1)	16 (10.1)	61 (53.0)	43 (37.4)	11 (9.6)
Nurk et al. [2004]	Case	9 (40.9)	10 (45.5)	3 (13.6)	NA	NA	NA
Norway	Control	6,598 (45.7)	6,332 (43.8)	1,522 (10.5)	NA	NA	NA
Pezzetti et al. [2004]	Case	57 (54.8)	36 (34.6)	11 (10.6)	56 (50.9)	46 (41.8)	8 (7.3)
Italy	Control	121 (41.9)	130 (45.0)	38 (13.1)	95 (32.9)	151 (52.2)	43 (14.9)

CLP: cleft lip with or without claft palate; **NA:** not available

Table 2 MTHFR Genotype Frequencies in CHD Studies

References		Frequency in mothers, n (%)			Frequency in children, n (%)		
C377T polymorphism	**Subjects**	**CC**	**CT**	**TT**	**CC**	**CT**	**TT**
Junker et al. [2001]	Case	NA	NA	NA	51 (44.7)	42 (36.9)	21 (18.4)
Germany	Control	NA	NA	NA	129 (56.6)	78 (34.2)	21 (9.2)
Wenstrom et al. [2001]	Case	NA	NA	NA	17 (65.4)	8 (30.8)	1 (3.8)
Alabama, USA	Control	NA	NA	NA	104 (89.6)	9 (7.8)	3 (2.6)
Storti et al. [2003]	Case	27 (26.2)	53 (51.5)	23 (22.3)	28 (27.2)	55 (53.4)	20 (19.4)
Italy	Control	52 (26.0)	108 (54.0)	40 (20.0)	52 (26.0)	108 (54.0)	40 (20.0)
Nurk et al. [2004]	Case	12 (48.0)	12 (48.0)	1 (4.0)	NA	NA	NA
Norway	Control	7,153 (49.5)	6,025 (41.7)	1,281 (8.8)	NA	NA	NA
Lee et al. [2005]	Case	NA	NA	NA	110 (51.6)	89 (41.8)	14 (6.6)
Taiwan	Control	NA	NA	NA	114 (58.4)	68 (34.9)	13 (6.7)
Shaw et al. [2005]	Case	NA	NA	NA	69 (45.1)	68 (44.4)	16 (10.5)
California, USA	Control	NA	NA	NA	180 (41.5)	202 (46.5)	52 (12.0)
Hobbs et al. [2006a]	Case	127 (46.2)	118 (42.9)	30 (10.9)	NA	NA	NA
Arkansas, USA	Control	48 (40.7)	56 (47.4)	14 (11.9)	NA	NA	NA
van Beynum et al. [2006]	Case	72 (45.6)	68 (43.0)	18 (11.4)	79 (47.9)	66 (40.0)	20 (12.1)
The Netherlands	Control	131 (50.2)	107 (41.0)	23 (8.8)	98 (44.5)	104 (47.3)	18 (8.2)
A1298C polymorphism		**AA**	**AC**	**CC**	**AA**	**AC**	**CC**
Storti et al. [2003]	Case	49 (47.6)	46 (44.6)	8 (7.8)	45 (43.7)	47 (45.6)	11 (10.7)
Italy	Control	101 (50.5)	86 (43.0)	13 (6.5)	101 (50.5)	86 (43.0)	13 (6.5)
Nurk et al. [2004]	Case	9 (36.0)	13 (52.0)	3 (12.0)	NA	NA	NA
Norway	Control	6,598 (45.7)	6,329 (43.8)	1,522 (10.5)	NA	NA	NA

CHD: congenital heart defects; **NA:** not available

Results

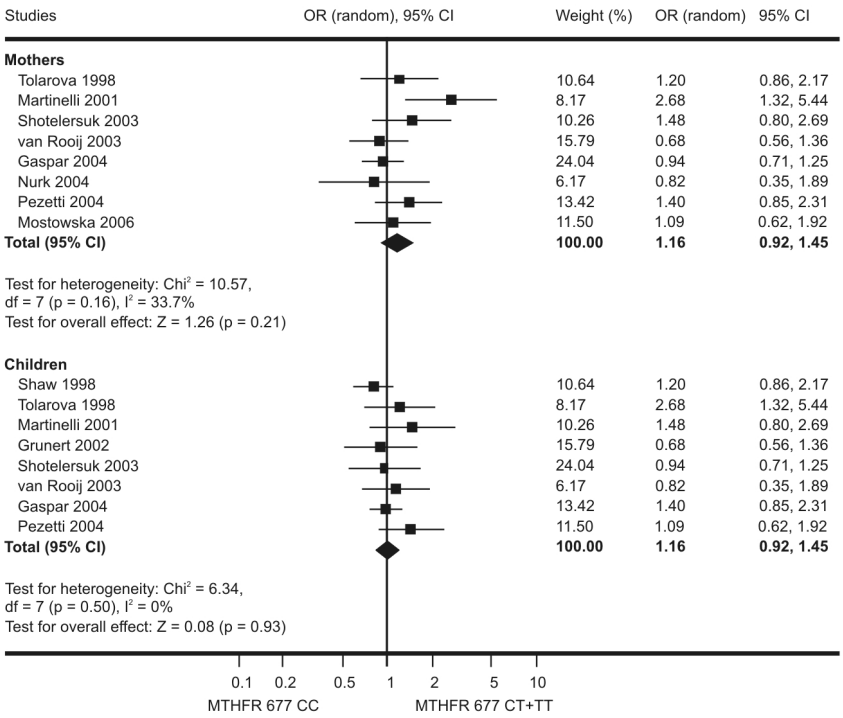
The included studies were all case-control studies, except for the cohort study of Nurk et al. (2004). In the CLP meta-analysis we included 263 mothers for tHcy data, 16,772 mothers and 3,341 children for the C677T polymorphism, and 15,584 mothers and 1,379 children for the A1298C polymorphism (Table I). For the CHD meta-analysis those numbers were 771, 15,599, 2,167, 14,777 and 303, respectively (Table II). The OR and 95% CI for the individual studies and the pooled analyses are presented in Figures 1–3. In all tHcy studies blood samples were drawn after an overnight fast with the exception of the study of Hobbs et al. (2005b). The pooled OR (95% CI) for maternal hyperhomocysteinemia and CLP was 2.3 (0.4–11.9), and 4.4 (2.6–7.3) for CHDs (Fig. 1). Based on the data provided in the study of Kapusta et al. (1999), an OR of 4.8 was computed instead of the OR of 5.1 that is mentioned in their article. Only two studies have investigated hyperhomocysteinemia in children with respect to CLP [32] and CHDs [35]. Therefore, we could not estimate a pooled OR for hyperhomocysteinemia in children with CLP or a CHD. The MTHFR genotype frequencies were consistent with HWE in all studies except for the group of casechildren in the study of Junker et al. (2001) and the control-children in the study of Wenstrom et al. (2001). The MTHFR 677CT/TT genotype and CLP revealed pooled ORs of 1.2 (0.9–1.5) in mothers and 1.0 (0.9–1.2) in children. For the MTHFR 1298AC/CC genotype these estimates were 1.0 (0.7–1.2) in mothers and 0.9 (0.6–1.2) in children, respectively (Fig. 2). In CHD-studies, the MTHFR C677T polymorphism showed a pooled OR of 1.0 (0.8–1.3) for the CT/TT genotype in mothers and 1.1 (0.9–1.5) in children. We considered excluding the studies of Junker et al. (2001) and Wenstrom et al. (2001), because the case and the control-group, respectively, were out of HWE. The CT/TT genotype in children then demonstrated a pooled OR of 1.0 (0.8–1.2). Two studies investigated the maternal MTHFR A1298C polymorphism in association with CHDs and the pooled OR was 1.2 (0.8–1.8) for the AC/CC carriers (Fig. 3). Only one CHD study reported of this polymorphism in children and showed an OR of 1.3 (0.8–2.1) [49]. The funnel plots were asymmetrical for tHcy concentrations in both CLP and CHD studies, the C677T polymorphism in CHD studies, and the A1298C polymorphism in CLP and CHD studies (data not shown).

Discussion

In this meta-analysis we used the results from studies published until September 2006 to calculate a pooled estimate of the reported associations between hyperhomocysteinemia, MTHFR polymorphisms and the risk of CLP and CHDs. Maternal hyperhomocysteinemia was significantly associated with a 4.4-fold increased risk of having a child with a CHD. This finding substantiates the hypothesis that maternal hyperhomocysteinemia is a risk factor for CHDs. The association was not significant for CLP, but no firm conclusion can be made based on the results of only two published studies. The point estimates for the MTHFR polymorphisms in mothers and children had small CIs and were not significant for both CLP and CHDs. These results suggest that the mutant MTHFR alleles do not independently contribute to the risk of a child with either CLP or a CHD.

Figure 2 Association between the MTHFR C677T (A) and A1298C (B) polymorphism in mothers and children and cleft lip with or without cleft palate

A



B

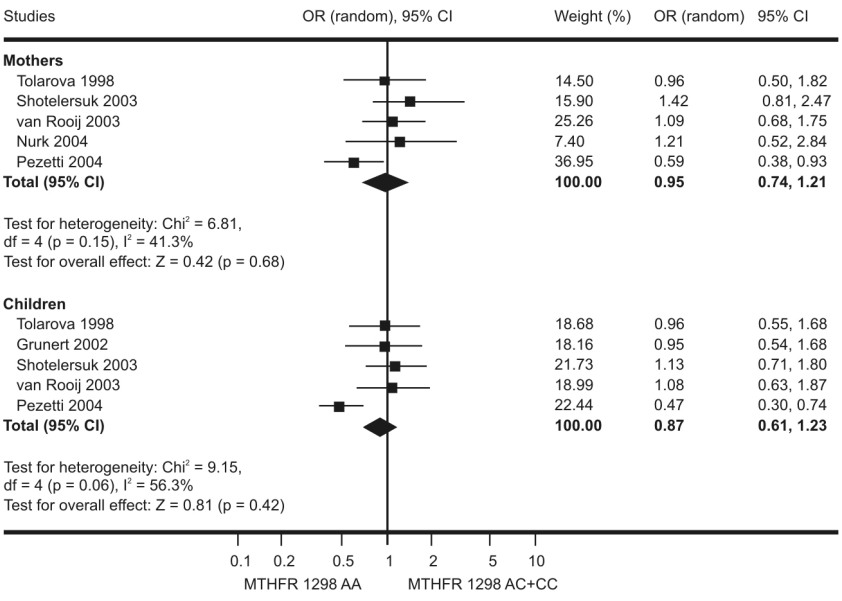
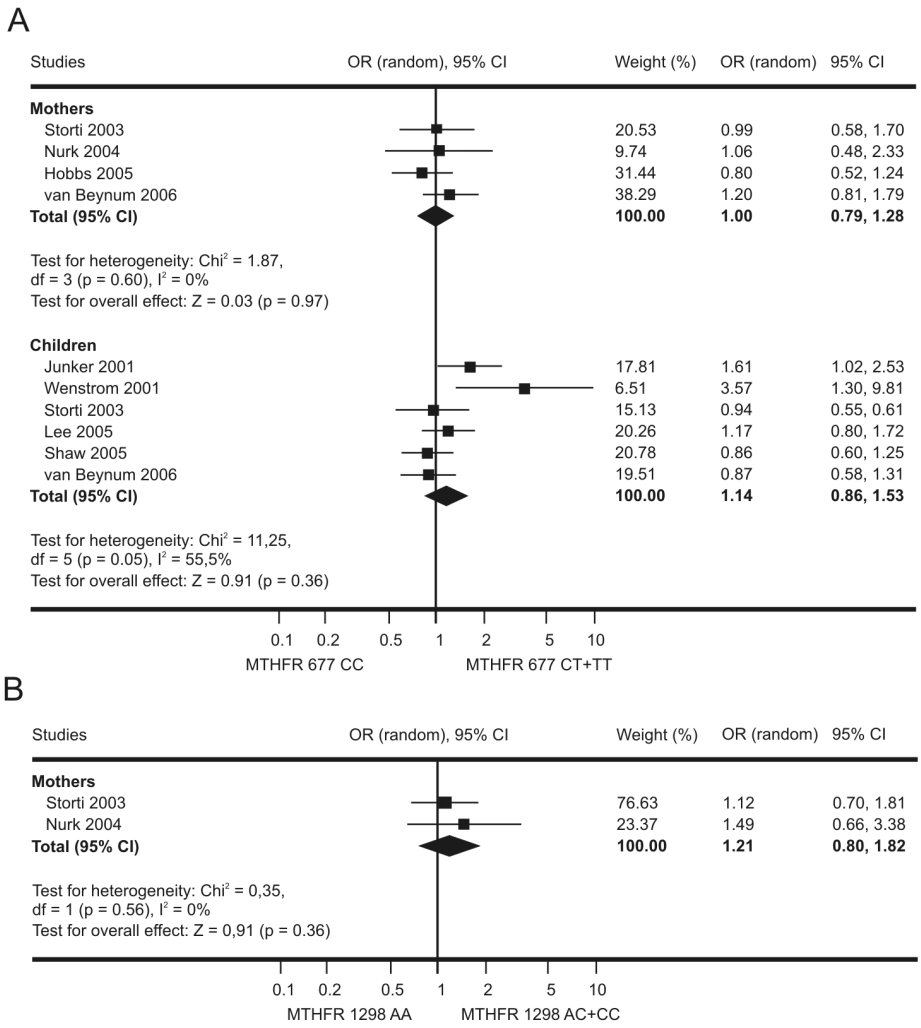


Figure 3 Association between the MTHFR C677T (A) and A1298C (B) polymorphism in mothers and children and congenital heart defects.



It is widely recognized that the origin of these congenital malformations is complex and multifactorial in nature, with genetic and environmental factors affecting various developmental pathways. Of interest, therefore, is that factors like diet and lifestyle can modify the effects of certain genetic polymorphisms. Heterozygosity for both MTHFR polymorphisms can result in a lower MTHFR activity than heterozygosity for either of the MTHFR mutations separately [11]. Two studies showed that tHcy concentrations were significantly higher in individuals with a combined heterozygosity for the MTHFR polymorphisms than those who were heterozygous for either the C677T or the A1298C genotype [11, 54]. Shotelersuk et al. (2003) demonstrated a significant increased risk of having a child with CLP if the mother was heterozygous for both MTHFR polymorphisms. The risk of a child with CLP [41] or a CHD [52] was only significantly increased if mothers, carrying

the MTHFR 677TT or MTHFR 1298CC genotype, also had a low periconceptional intake of dietary folate and/or folic acid supplements. These examples of gene–gene and gene– nutrient interactions explain why neither of the two MTHFR polymorphisms is an independent risk factor for CLP and CHDs. Moreover, these previously published articles give insight in the protective effect of maternal use of multivitamins containing folic acid in the periconceptional period on the risk of having a child with CLP [3] or a CHD [4-5, 55]. Dietary intake and use of B-vitamin supplements can compensate for the reduced activity of the MTHFR enzyme and lower tHcy concentrations, thereby decreasing the risk of these congenital defects. We have to consider some strengths and limitations of this meta-analysis. The use of a meta-analysis can overcome the low power of small sample size studies, and, therefore, reconcile previously conducted studies with inconsistent results. Although, the number of included tHcy studies in this metaanalysis is quite low for both congenital defects, all tHcy concentration measurements were performed by the high performance liquid chromatography method. The risk estimates for maternal hyperhomocysteinemia in CLP and CHDs are consistent and, therefore, laboratory errors are not likely. In the pooled analysis of hyperhomocysteinemia in mothers and CLP risk only two studies were included with evidence of heterogeneity. This heterogeneity might be caused by the pilot study of Wong et al. [1999]. In this study the cutoff value of the 97.5th percentile was used instead of the 90th percentile that was used by the other tHcy studies, resulting in a low number of hyperhomocysteinemic mothers. Moreover, they included both methionine afterload and fasting tHcy concentrations. With concern to the genotyping of MTHFR polymorphisms, laboratory errors are not likely because the genotyping has been done using standard protocols with polymerase chain reactions and restriction enzyme digestion in all studies. Moreover, all studies showed genotype frequencies that were consistent with HWE for both cases and controls with an exception of the cases in the study of Junker et al. [2001] and the controls in the study of Wenstrom et al. [2001]. The study of Wenstrom et al. in particular was the source of heterogeneity in the analysis of CHDs and the MTHFR C677T genotype in children, possibly because their control-group was out of HWE. We considered exclusion of both studies, but the point estimates did not substantially change. The analysis of CHDs and MTHFR C677T polymorphisms in children, and the analysis of CLP and A1298C polymorphisms in children demonstrated P values less than 0.10 for the Q test for heterogeneity (Figs. 2 and 3). We used the random effects model in all analyses, thereby accounting for the heterogeneity. In addition, the point estimates of the fixed and the random effects model are nearly identical, which suggests that heterogeneity is not a big issue. Differences in risk estimates for MTHFR polymorphisms can also be caused by etiologic heterogeneity between populations, geographical variations of the studied populations, different selection of controls or even by the folate intake of the population [56]. The 677TT and 1298CC genotype frequencies of the included studies demonstrate the known geographical variations [55, 57-59]. Regarding the selection of controls, most studies included unrelated and unaffected controls. We considered the influence of the studies that used another selection of controls, but these studies did not significantly alter the point estimates. Publication bias has to be addressed in metaanalyses because it can be a substantial cause of bias. The funnel plots are asymmetrical for the A1298C polymorphism and tHcy concentration in both CLP and CHDs, and the C677T polymorphism in CHDs

suggesting publication bias towards overestimation of the results. Asymmetry might be due to the low number of published studies. Furthermore, asymmetric funnel plots can also be caused by language and citation bias, if studies with non-significant results are published in non-English languages, and are thereby less likely to be cited. However, the studies with non-significant findings have also been included in the analyses, thereby reducing the chance of publication bias. In conclusion, we demonstrated in this metaanalysis that maternal hyperhomocysteinemia is a risk factor for CHDs. The MTHFR polymorphisms C677T and A1298C did not show to be significantly associated with CLP or CHDs. Further research should be performed to investigate the interactions between maternal hyperhomocysteinemia, B-vitamin intake, related polymorphisms and the risk of CLP and CHDs.

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CHAPTER 3

THE RISK OF HAVING A CHILD WITH A CLEFT LIP WITH OR WITHOUT PALATE AND THE MATERNAL ABCB1 C3435T POLYMORPHISM AND MEDICATION USE

Bart JB Bliek, Ron HN van Schaik, Ilse P van der Heiden, Fakhredin A Sayed Tabatabaei, Cock M van Duijn, Eric AP Steegers, Régine PM Steegers-Theunissen and the Eurocran Gene-Environment Interaction Group
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Abstract

Gene–environment interactions in the periconceptional period play an increasing role in the pathogenesis of birth defects, including cleft lip and/or cleft palate (CL/P). The P-glycoprotein, encoded by the ABCB1 gene, is suggested to protect the developing embryo from medication and other xenobiotic exposures. Furthermore, maternal medication use during early pregnancy is a significant risk factor for CL/P offspring. Therefore, the aim of this study is to investigate the association between the maternal and child's functional ABCB1 3435C>T polymorphism, periconceptional medication exposure, and the risk of a child with CL/P. A case–control study was performed among 175 mothers and 98 of their children with CL/P and 83 control mothers and their 65 children. Information on medication and folic acid use was collected. Mothers carrying the 3435TT genotype and using medication showed a 6.2-fold (95%CI 1.6–24.2) increased risk of having a child with CL/P compared to mothers carrying the 3435CC genotype and not using medication. Periconceptional folic acid use reduced this risk by approximately 30% (OR 3.9, 95%CI 0.9–18.0). Mothers carrying the 3435TT genotype, using medication and not taking folic acid showed the highest risk estimate (OR 19.2, 95%CI 1.0–369.2). These data suggest that mothers who carry the ABCB1 3435C>T polymorphism are at significantly increased risk for having offspring with CL/P, especially mothers using medication in the periconceptional period.

Introduction

A nonsyndromic cleft lip with or without cleft palate (CL/P) is a complex congenital malformation, in which gene–environment interactions play an increasing role [1]. Because during pregnancy the mother is the environment of the child, maternal exposures together with genetic vulnerabilities of the mother and/or child are of interest. The best examples in this regard are the combined exposures of maternal folic acid use, the 677C>T polymorphism in the MTHFR gene in association with CL/P [2-5]. In addition, interesting associations are shown between the MSX1, GSTT1, and CYP1A1 genes and smoking [6-7]. Medication use during the period of embryogenesis is also associated with a higher CL/P risk, in particular for corticosteroids [8] and antifolates [9]. Several medications are substrates for P-glycoprotein (P-gp), an efflux pump encoded by the ABCB1 gene (old nomenclature: MDR-1, PGY1, CD243). P-gp protects the cell from harmful exposures by actively exporting various substrates across the cell membrane. The 3435C>T synonymous single nucleotide polymorphism of ABCB1 is associated with an increased degradation of the ABCB1 mRNA and consequently a decreased P-gp expression [10-11]. This suggests that this polymorphism may modify cellular exposures to medication [12]. Based on this background information, we hypothesized that the ABCB1 3435C>T polymorphism and periconceptional exposure to medications increases the risk of CL/P. We investigated this hypothesis in Dutch Caucasian mothers and their childrenwith CL/ P and in control mothers and their children, taking folic acid intake into consideration, a factor believed to reduce the risk of CL/P and a potential modifier of the teratogenicity of certain medications [9].

Materials and methods

Study Population

The study population and design were described previously [13]. Briefly, between 1998 and 2003, a case–control study was conducted by nine of the largest cleft palate teams in the Netherlands. We recruited mothers and their child with a nonsyndromic CL/P and healthy control mother child pairs. In each hospital team, the CL/P of the child was diagnosed by a clinician according to a standard registration form developed by the Dutch Association for Cleft Palate and Craniofacial Anomalies. The standardized registration was performed when the child was approximately 15 months of age. Most associated malformations and developmental delays are identified in the first year of life, which is important in selecting case and control children. The unrelated control children did not have major congenital malformations and were approximately 15 months of age. They were enrolled from the same population as the case group by approaching the mothers with posters and leaflets in child healthcare centers, which are part of the Dutch Healthcare system. All participants were Dutch Caucasians and none of the children was adopted. The study was approved by the Central Committee for Human Research in The Hague, The Netherlands and by the Medical Ethical Committees of all participating hospitals. Written informed consent was obtained from every participant and on behalf of their child before entering the study. At 15 months after delivery, detailed information on maternal periconceptional medication and folic acid use, including brand, type, amount, frequency, and precise duration of exposure, was obtained using validated questionnaires, which were filled out at home. All questionnaires were checked by the researcher for completeness and inconsistencies. Mothers were considered medication and folic acid users when taking any medication or any tablet containing 0.4–0.5 mg folic acid in a single or multivitamin preparation daily in the periconceptional period, that is, between 4 weeks before until 8 weeks after conception. This is the recommended period for folic acid use by the Dutch government for all women who are planning pregnancy. On average, folic acid was used from 9.7 weeks before until 12.6 weeks after conception. At the same time blood samples were obtained for DNA isolation. From this database we randomly selected the DNA samples of 175 mothers and their children with CL/P for whom DNA was available (98 children) and of 83 control mothers and their children for whomDNAwas available (65 children) and genotyped them for the ABCB1 3435C>T polymorphism (RS1045642). The method employed was restriction fragment length polymorphism analysis [14].

Analysis

General characteristics were compared by using the Chi-squared test or Student's t-test. Univariate and multivariate binary logistic regression analyses were used to estimate odds ratios (ORs) with 95% confidence intervals (95%CI). Modification of the CL/P risk by medication and folic acid use and the ABCB1 genotype was determined by ordinal logistic regression, in which adjustments were made for educational level, family history of clefts, and periconceptional alcohol use. Statistical analysis was done using SPSS software version 12 (SPSS, Inc., Chicago, IL). P values of 0.05 or less were considered statistically significant.

Results

Characteristics

In Table I, the demographics of the mothers and children are given. The case mothers were approximately 2 years younger (P<0.05) and had a lower educational level (P<0.01) than controls. Periconceptional medication use was significantly higher among mothers of children with CL/P (36.6%) compared to control mothers (20.5%, P<0.01). In Table II, the medication used by case and control mothers is presented. It included corticosteroids, antibiotics, anti-epileptics, analgesics, anxiolytics, antipsychotics, antidepressants, antihistamines, and fungicides. Mothers of a child with CL/P used periconceptional folic acid less often (57.7%) compared to controls (75.9%, P<0.005). Maternal smoking was comparable between the groups, but alcohol use was significantly lower in case mothers (P=0.003). In addition, case mothers had more relatives with a cleft (P<0.001) and more boys were affected with CL/P (P<0.05).

Table 1 General characteristics of the case and control mother-child pairs

Characteristics	CL/P[a]	Control
Mothers, N	175	83
Age at delivery of index pregnancy, years mean (range)	30.4 (16.5 – 42.1)*	32.4 (21.9 – 41.9)
Duration index pregnancy, weeks mean (range)	40.0 (28.0 – 42.7)	40.0 (34.1 – 41.9)
Educational level		
Low, N (%)	41 (23.4)***	4 (4.8)
Intermediate, N (%)	76 (43.4)	23 (27.7)
High, N (%)	58 (33.1)	56 (67.5)
Spontaneous abortion, N (%)	53 (30.3)	18 (21.7)
Previous stillborn, N (%)	3 (1.7)	0 (0.0)
Congenital malformations		
Mothers with congenital malformation, N (%)	18 (10.3)**	2 (2.4)
Family (1st and 2nd degree) history of clefts, N (%)	40 (22.9)***	4 (4.8)
Periconceptional exposure		
Medication, N (%)	64 (36.6)***	17 (20.5)
Folic acid, N (%)	101 (57.7)***	63 (75.9)
Multivitamins, N (%)	34 (19.4)	15 (18.1)
Smoking, N (%)	45 (25.7)	16 (19.3)
Alcohol, N (%)	63 (36.0)***	47 (56.6)
Children, N	98	65
Gender, boy, N (%)	61 (62.2)***	27 (42.2)
Birth weight, grams mean (range)	3,370 (1,361–4,765)	3,340 (2,500–4,570)

[a]CL/P, cleft lip with or without cleft palate.
* *t*-test, P<0.05.
**X^2 test, P<0.05.
*** X^2 test, P<0.01.

ABCB1 C3435T

The distribution of the maternal ABCB1 genotype for 3435CC/CT/TT genotypes in case mothers was 20.0%, 49.1%, and 30.9% in case mothers, with a T-allele frequency of 55%, and 25.3%, 53.0%, and 21.7% in control mothers, with a T-allele frequency of 48% (Table III). Among children with CL/P, the distributionwas 25.5%,

49.0%, and 25.5%, with a T-allele frequency of 50%, and among control children the distribution was 29.2%, 44.6%, and 26.2%, with a T-allele frequency of 48% (Table III). Both case and control genotypes were in Hardy–Weinberg equilibrium.

Table 2 Periconceptional medication use of case and control mothers with the number of mothers who used the medication in parentheses

Medication	Case mothers (n=64)	Control mothers (N=17)
Analgesics	acetaminophen (12), diclofenac (1)	acetaminophen (1)
Antacids	aluminium hydroxide magnesium carbonate (1), ranitidine (1)	-
Antibiotics	nitrofurantoin (5), amoxicillin (4), sulfonamides (4), ciprofloxacin (2), tetracycline (1), erythromycin (1), norfloxacin (1)	amoxicillin (2), nitrofurantoin (2)
Antidiarrhoica/ obstipation	loperamide-oxide (2), domperidon (2), buscopan (1)	-
Anti-epileptics	carbamazepine (1)	-
Antihistamines	meclizine (1), cetirizine (1)	cyclizine (1)
Antipsychotics	perphenazine (2)	-
Anxiolytics/ antidepressants	diazepam (2), fluoxetine (1), fluvoxamine (1), venlafaxine (1)	paroxetine (2)
Corticosteroids	hydrocortisone (2), prednisone (1), budesonide (2), beclomethasone (1)	fluticasone (1)
Fungicides	metronidazole (4), ketoconazole (3), clotrimazole (2), itraconazole (1)	clotrimazole (2), miconazole (1)
Hormones	clomiphene (3), GnRH analog (2), progesterone (2), levothyroxine (2), insulin (2), FSH/LH (1), norethindrone (1)	GnRH analog (2), FSH/LH (1),clomiphene (1), GnRH antagonist (1)
Hypertension	methyldopa (2), losartan (1)	-
Pulmonary function	salbutamol (7), ipratropium (1), formoterol (1)	salbutamol (1)
Thrombocyte aggr. inhibitor	acetylsalicylic acid (2)	acetylsalicylic acid (1), heparin (1)
Others	xylometazoline (3), bromhexine (1), gamma globulins (1), ergotamine (1), prochlorperazine (1), naratriptan (1), betahistine (1)	hypromellose (1), xylometazoline (1)

Periconceptional Medication Exposure and ABCB1 C3435T

Any maternal medication use resulted in an adjusted OR of 2.2 (95%CI 1.2–4.1) for CL/P risk after correction for educational level, family history for CL/P and alcohol use. The maternal ABCB1 3435TT genotype together with periconceptional medication use showed a 6.2-fold (95%CI 1.6–24.2) increased risk of CL/P with a significant P for trend of 0.01 (Table III). Increased CL/P risks were observed in children (although these results were not statistically significant) who were carriers of the 3435CT and the 3435TT genotype in combination with maternal periconceptional medication use, that is, 3.9 (95%CI 0.9–16.1) and 2.5 (95%CI 0.7–9.5), respectively, with a significant trend, P=0.02 (Table III).

Folic Acid Use

Maternal periconceptional folic acid use decreased the risk for CL/P after adjustment for educational level, family history of CL/P, and alcohol use (OR 0.5, 95%CI 0.3–0.9). A 19.2-fol d increased risk (95%CI 1.0–369.2) for having a

child with CL/P was demonstrated for mothers with the 3435TT genotype who had been using medication and did not use folic acid. For mothers who used folic acid, the increased risk for having a child with CL/P associated with medication use in combination with the 3435TT genotype decreased by approximately 30% (OR 3.9, 95%CI 0.9–18.0). These results suggest that folic acid use modified the association between the maternal ABCB1 polymorphism and medication use and the risk of CL/P offspring.

Table 3 Distributions and risk estimates of the ABCB1 3435C>T polymorphism for mothers and children

ABCB1 3435 C>T	Pts, n(%)[a]	Ctrls, n(%)[b]	OR (95%CI)[c]	Medication use	Pts [a]	Ctrls [b]	OR (95%CI) [c]	P for trend
Mother				**Mother**				
TT	54	18	1.8	Yes	23	3	6.2 (1.6–24.2)*	
	(30.9)	(21.7)	(0.8–3.8)	No	31	15	1.7 (0.7–4.1)	
CT	86	44	1.2	Yes	27	10	2.2 (0.8–5.7)	
	(49.1)	(53.0)	(0.6–2.3)	No	59	34	1.4 (0.7–3.0)	
CC	35	21	1.0	Yes	14	4	2.8 (0.8–10.2)	
	(20.0)	(25.3)	(ref.)	No	21	17	1.0 (ref.)	0.01
Children				**Mother**				
TT	25	17	1.1	Yes	13	4	2.5 (0.7–9.5)	
	(25.5)	(26.2)	(0.5–2.6)	No	12	13	0.7 (0.3–2.1)	
CT	48	29	1.3	Yes	15	3	3.9 (0.9–16.1)	
	(49.0)	(44.6)	(0.6–2.7)	No	32	25	1.0 (0.4–2.4)	
CC	25	19	1.0	Yes	7	5	1.1 (0.3–4.2)	
	(25.5)	(29.2)	(ref.)	No	18	14	1.0 (ref.)	0.01

The risk estimates are stratified for maternal periconceptional medication use.
*P<0.05. [a] Patients, [b] Controls, [c] Odds ratio (95% confidence interval).

Discussion

This is the first study showing that the ABCB1 3435TT genotype results in a sixfold increase in the risk of having a child with CL/P in mothers using medication periconceptionally. P-gp plays a role in the regulation of the exposure of the embryonic tissues to medicines. The ABCB1 3435TT genotype lowers P-gp activity, and as such increases the exposure to medication, which could detrimentally affect embryogenesis. The observed tendency of the higher CL/P risk (P=0.02) among carriers in children in combination with periconceptional medication exposure may suggest that the child's P-gp expression is also involved in its protection against toxic exposures.

We considered mothers as medication users if they had used any medication during the periconceptional period. The effect of the ABCB1 3435TT genotype might therefore even be stronger when only known P-gp dependent medicines are taken into consideration. However, for many drugs it is yet unknown whether they are a substrate for P-gp and this would require a very large study population.

Of interest is that periconceptional folic acid use appears to mitigate the increased risk for CL/P by the maternal ABCB1 3435TT-medication interaction. This is in line with the results of Hernandez-Diaz et al. (2000), who reported that the increased risk of having offspring with CL/P associated with the use of dihydrofolate reductase inhibitors was reduced in mothers who also used folic acid.

It cannot be excluded that there are also direct effects of folic acid on the ABCB1 expression or efflux pump capacity, comparable to the earlier reported increase of the functional capacity of the related transporter ABCC1 by folic acid [15].

Of interest is that the combined effect of both infant and mother possess the at-risk genotype with and without exposure. However, the numbers were too small for further analysis and therefore, future studies should help to determine the relative contributions of maternal and infant detoxification with respect to medication use during pregnancy.
To assess the validity of the study, some methodological issues need to be considered. To minimize recall bias of the periconceptional exposure data derived from mothers and to increase the comparability of the case and control children, we used a standardized time of 15 months after delivery for questionnaire completion for cases and controls. Cases and controls were largely derived from the same population and were homogeneous with regard to ethnic background and geographic region. To minimize misclassification, CL/P was diagnosed at specialized cleft centers and according to the standards of the Dutch Association for Cleft Palate and Craniofacial Anomalies. The educational level of the mothers of the control group was higher than that of the case mothers. Because a higher educational level is in general associated with a healthier lifestyle and more folic acid and less medication use, it may have confounded the associations. For that reason we adjusted for education, which did not significantly affected the risk estimates. Therefore, selection bias and confounding by educational level seems unlikely. The retrospective character of the case–control design makes the exposures of medication and folic acid use sensitive to recall bias. Mothers with adverse pregnancy outcome may be more prone to recall bias, which may have influenced risk estimates. Several of the reported medications, such as corticosteroids, are teratogenic in experimental and human studies; however, confounding by indication cannot be excluded. Therefore, large scale databases on the use of prescribed medication and obstetrical databases should be used to determine whether the observed associations are due to maternal medication use, the underlying maternal disease, or both.
Because the association of theABCB1 3435TT genotype with CL/ P was only observed in mothers using medication, we are not recommending preconceptional screening of the general population for this genotype to prevent CL/P. However, after confirmation of these results in much larger populations, selective genotyping of mothers using medication preconceptionally might contribute to improved outcomes.

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CHAPTER 4

MATERNAL FOLATE RECEPTOR AUTOANTIBODIES AND CLEFT LIP AND/OR PALATE

Bart JB Bliek, Sheldon P Rothenberg, Régine PM Steegers-Theunissen
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Abstract

For a long time it is known that maternal periconceptional folic acid supplementation reduces the incidence of congenital anomalies such as neural tube defects and non-syndromic cleft lip and or cleft palate (CL/P). Recently it has been shown that autoantibodies against the folate receptor are present in mothers who gave birth to a child with neural tube defects. Therefore we hypothesize that this antiserum also occurs in women with a child with CL/P. Standardized blood samples from 11 mothers with an CL/P affected child and 10 control mothers were taken at 15 months after the index pregnancy and autoantibodies against the folate receptor, serum folate, erythrocyte folate, serum homocysteine and vitamin B12 were measured. Questionnaires about lifestyle, food- and vitamin supplement intake were filled in by all mothers. Antiserum was found in 9 of the 11 case mothers, and in 3 of the 10 control mothers ($p=0.03$). Furthermore, from the women with antiserum, all except one had give birth before, in contrast to the women without antiserum, from which 3 of 9 had had children before ($p=0.02$). We conclude that serum from mothers with a CL/P child contains autoantibodies against the folate receptor. Whether the presence of this antiserum is a causal factor for CL/P is subject for further research.

Introduction

Cleft lip and/or palate (CL/P) is a frequent birth defect in the Netherlands with a birth prevalence rate of 1 to 2 per 1,000 life births from which the multifactorial etiology is not fully understood. Various association studies reported increased CL/P risks with health- and life style factors, such as medication and alcohol use, smoking[4], low socio-economic status[1] en low educational level of the mother. However, evidence is accumulating that, alike the risk on having a child with a neural tube defects, low intake of food folates or folic acid supplements in early pregnancy is related to increased CL/P risk (5,9,11 recente artikelen search voor uitvoeren(10, Shaw et al., Itikala et al. 2001, etc idem)). Still, a large part of the women pregnant of a child with CL/P do not show a severe folate deficiency. This is partly explained by maternal mutations of folate metabolism genes, such as the TT variant of the methylene tetrahydrofolate reductase-gene resulting in reduced folate availability and hyperhomocysteinemia, and associated with increased CL/P risk [1].

The availability of folate is regulated by folate receptors [2] and carriers [3], present on cell membranes. Knock-out animal studies showed that folate receptors and carries are vital for embryonic development and viability [4-5] and it is thus likely that the folate transporters are equally important in human embryogenesis. Several studies reported mutations of the folate receptors in association with the risk of neural tube defects [6-7], though up to date this could not be confirmed in larger populations, nor be associated with CL/P risk. Therefore, of great interest is the recently found antiserum against the homologue of the human folate receptor α gene that induces embryo lethality and congenital malformations, such as neural-tube and heart defects and cleft palate [8] . This antiserum, is capable of blocking the folate receptor [9] and may contribute to a low intracellular folate status.

From this background we hypothesize that a compromised maternal folate status may be due to presence of folate receptor autoantibodies in mothers to be and

thus might be related to increased CL/P risk.

Materials and Methods

As part of a nationwide case-control triad study on nonsyndromic CL/P conducted between 1998-2000 in the Netherlands [1], we randomly selected 11 mothers with nonsyndromic CL/P offspring and 10 mothers with healthy offspring without structural congenital malformations. This study has been performed with informed consent from every participant and has been approved by the Medical Ethical Boards of the participating hospitals. Around fourteen months after the index-pregnancy questionnaires were filled out by the mothers and verified by the researcher during the hospital visit. The questionnaires comprised information on maternal demographics, exposures, vitamin supplement intake, medication and illnesses. At the same visit standardized maternal venous blood samples were taken. After pre-treatment the biochemical determinations were carried out and the remnant sera were stored at -80C until further process.

Laboratory determinations

The remnant sera were encoded to ensure blinded measurement and autoantibodies against the folate receptor were determined by incubation with human placental folate receptors radiolabeled with [^{3}H]folic acid as described before [9]. The intra-and interassay coefficients of variations (CV) were ≤3% and ≤ 5%, respectively.
Red blood cell and serum folate were determined by a microbiological assay of which the intra- and interassay CV were 6.1% and 10.2% respectively [10]. Plasma total homocysteine was determined with an automated HPLC method with reverse phase separation and fluorescence detection and intra- and interassay CV were ≤ 6.5% [11].

Statistical analysis

Presence of folate receptor autoantibodies in the selected mothers was compared with chi-square statistics and demographic and biochemical variables were compared with the nonparametric Wilcoxon two sample test using SPSS software. P values of 0.05 or less were considered significant.

Results

The demographic data from case and control mothers and their children are presented in table 1. The case group showed a higher frequency of periconceptional medication use ($p=0.04$) at the moment of investigation. Case mothers demonstrated significantly higher median tHcy concentrations compared with controls, i.e. 12.0 (10.0-69.0) and 10.4 (8.9-13.4) µmol/L ($p<0.01$, table 1). No significant difference was found in the serum and RBC folate concentrations. Unexpectedly, serum from 9 out of 11 (82%) case-mothers with CL/P offspring and 3 out of 10 (30%) controls contained autoantibodies against folate receptors ($p=0.03$). The median (range) of the autoantibody concentrations was 0.55 (0-0.75) and 0 (0-0.64) in case and control mothers respectively.

Comparison of mothers with and without folate receptor autoantibodies showed a higher rate for earlier life borns (p=0.005) in antibody positive mothers. Biochemically, the median serum folate concentrations were significantly lower (p=0.05) and tHcy concentrations were significantly higher (p=0.05) in antibody positive mothers (table 1). The RBC folate concentrations were not significantly different.

Table 1. Maternal characteristics and biochemistry in medians (ranges) and numbers (percentages)

Mothers	Cases (n=11)	Controls, (n=10)	Antibodies present (n=12)	Antibodies absent (n=9)
Age at delivery, y (median)	33.8 (27.9-38.7)	32.9 (22.4-35.7)	33.8 (27.9-38.7)	32.2 (22.4-36.2)
Educational level §				
Low, n (%)	6 (54.5)	5 (50.0)	7 (58.3)	4 (44.4)
High, n (%)	5 (45.5)	5 (50.0)	5 (41.7)	5 (55.6)
Parity, n (%)	9 (81.8)	5 (50.0)	11 (91.7)	3 (33.3)*
Miscarriages, n (%)	4 (36.4)	3 (30.0)	4 (33.3)	3 (33.3)
CLP family history, n (%)	1 (9.1)	0 (0)	1 (8.3)	0 (0)
Periconceptional use of:				
Folic acid, n (%)	2 (18.2)	3 (30.0)	3 (25.0)	2 (22.2)
Medication, n (%)	7 (63.6)	2 (20.0)*	7 (58.3)	2 (22.2)
Antiserum concentration[#] (dpm x 10^4/mL serum)	0.60 (0.49-0.75)	0.56 (0.46-0.64)	0.58 (0.46-0.75)	NA
Serum folate (nmol/L)	14.0 (5.0-22.0)	17.2(7.9-54.6)	13.0 (5.0-17.0)	22.0 (7.9-54.6)*
RBC folate (nmol/L)	832 (227-1183)	614(454-1398)	541(227-1174)	793(454-1398)
Plasma tHcy (µmol/L)	12.0 (10.0-69.0)	10.4(8.9-3.4)*	12.0(10.0-69.0)	10.4 (9.0-16.0)*

Reference values for serum folate, RBC (red blood cell) folate and plasma (tHcy) total homocysteine are 7.1—40.0nmol/l, 295—800nmol/l and 8—19µmol/l, respectively.
[a] Low: Primary/lower vocational/intermediate secondary/intermediate vocational education. High: High vocational and academic education.
[b] Concentration in antiserum positive mothers.
* p<0.05.

Discussion

These data indicate that autoantibodies against folate receptors are frequently present in mothers of a child with CL/P. These antibodies show a non-differential inhibition of folate transport in placenta tissue, indicating they may cause reduced intracellular availability of folate. Low intake of food folates and folic acid supplements around conception is associated with an increased CL/P risk. It is therefore likely that maternal folate receptor autoantibodies contribute to the CL/P risk, comparable to the neural tube defects risk [9]. This is substantiated by the biochemical findings of low serum folate and high homocysteine concentrations in the presence of autoantibodies. In addition, the biochemical results fit with the in vitro findings of a reduced cellular folate uptake.
Mechanistically these findings raise the question if there is a common pathway in the pathogenesis of neural tube defects and CL/P. If folate receptor autoantibodies increase the risk of both congenital malformations, than theoretically they should be seen together often, which actually is not the case. It is also not likely that the antibodies are tissue specific, not only because both tissues express the same folate receptors, but also because in principle the placenta is impermeable for maternal autoantibodies. A temporal exposure to the antibodies is evenly unlikely

since in general autoantibodies do not disappear quickly and palatal development rapidly follows neural tube closure. These contradictions require further pathway and malformation specific exploration.
The folate receptor autoantibodies do not affect the reduced folate carrier. This transporter has high affinity for synthetic folic acid, reduced and methylated in vivo. From this we may hypothesize that the autoantibody-mediated blocking of cellular folate uptake by folate receptors could be circumvented by intake of additional synthetic folic acid [2]. Another mechanism that is in line with our findings is that the folate receptor has a higher affinity for folic acid which may displace an autoantibody with lower affinity for the receptor. These hypotheses are subject for future research.
The mechanism by which folate receptors might become self antigens is not known. In this set of data the antibody positive mothers showed a significantly higher rate of earlier life borns compared to antibody negative mothers. Autoimmunity may be induced by epitopes of the folate receptors exposed as a result of injury and proteolysis of the reproductive tissues, which together with maternal genetic factors may trigger the generation of autoantibodies. Our data however, do not suggest that mothers carrying antibodies more often suffer from autoimmune disease.
This study was designed as a pilot study, therefore the sample size is small and the data should be interpreted cautiously. On the other hand, the strengths of our study is the homogeneity of the groups, the accurateness of the CL/P diagnosis, the equal distribution of the demographic and clinical characteristics of both groups, the systematic recruitment and random selection of case and control mothers, the standardized blood sampling and storage at a fixed moment 15 months after delivery, and the blinding of the samples before determination of the antibodies in the sera. Therefore, it is very unlikely that our data are confounded by selection bias.
In conclusion, we have identified an autoantibody against the folate-receptor membrane protein in mothers of CL/P offspring. Whether the association between maternal autoantibodies against folate receptors and nonsyndromic CL/P offspring reflects a causal relation has to be further investigated.

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PART THREE

BIOLOGICAL STUDIES

CHAPTER 5

GENOME-WIDE PATHWAY ANALYSIS OF FOLATE-RESPONSIVE GENES TO UNRAVEL THE PATHOGENESIS OF OROFACIAL CLEFTING IN MAN

Bart JB Bliek, Régine PM Steegers-Theunissen, Leen Blok, Lindy Santegoets, Jan Lindemans, Ben Oostra, Eric AP Steegers, Annelies de Klein
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Abstract

BACKGROUND: A cleft of the lip with or without the palate (CL/P) is a frequent congenital malformation with a heterogeneous etiology, for which folic acid supplementation has a protective effect. To gain more insight into the molecular pathways affected by natural folate, we examined gene expression profiles of cultured B-lymphoblasts from CL/P patients before and after the addition of 5-methyltetrahydrofolate (5-mTHF) to the cultures. METHODS: Immortalized B-lymphoblasts from five children with CL/P were cultured in folate-deficient medium for 5 days. 5-mTHF was added to a concentration of 30 nM. Gene expression patterns were then evaluated before and after supplementation using Human Genome U133 Plus 2.0 arrays. Data analysis was performed with Omniviz and the GEPAS analysis suite. Differential genes were categorized into biological pathways with Ingenuity Pathway systems. Differential expression was validated by quantitative RT-PCR. RESULTS: Using supervised clustering, with a false discovery rate <1%, we identified 144 and 409 significantly up-regulated and down-regulated probesets, respectively, after 5-mTHF addition. The regulated genes were involved in a variety of biological pathways, including one carbon pool and cell cycle regulation, biosynthesis of amino acids and DNA/RNA nucleotides, protein processing, apoptosis, and DNA repair. CONCLUSIONS: The large variety of the identified folate responsive pathways fits with the modifying role of folate via the methylation pathway. From the present data we may conclude that folate deficiency deranges normal cell development, which might contribute to the development of CL/P. The role of these folate responsive genes in CL/P development is intriguing and needs further investigation.

Introduction

Clefting of the lip with or without the palate (CL/P) is a common congenital malformation that occurs in approximately 14.2 per 10,000 live births in the Netherlands [1]. The etiology of CL/P is largely unknown, but is considered multifactorial in origin. As recently reviewed by Krapels et al. [2] associations between CL/P and developmental genes, such as TGFa and MSX1, and linkage disequilibrium of various chromosomal regions, such as 3p21.2, 10p13, and 16p13.3 and CL/P emphasize the involvement of genetic components. Of great interest are the findings of the last two decades that environmental factors play a role in CL/P etiology as well. Maternal periconception use of folic acid supplements and folate-rich food [3-4], medication [5], and smoking [6-7] are known to modulate the risk of having a CL/P child. We have shown that periconception supplementation with folic acid reduces the CL/P birth prevalence rate by approximately 50% [4], thereby supporting the recommendation of folic acid supplementation in the periconception period, not only to prevent neural tube defects (NTDs) but CL/P as well. Other countries have started folic acid fortification programs with beneficial effects on the reduction of NTDs [8-9]. Such programs imply long-term exposure of the total population to folate supplements. It is therefore remarkable that studies investigating the effects of synthetic folic acid and natural folate on biological processes are very scarce [8]. After reduction of folic acid to natural folates, folate derivatives serve intracellularly as a one-carbon group donor for the synthesis of purines, pyrimidines, and proteins and the remethylation

of homocysteine into methionine. The methionine metabolite, S-adenosylmethionine, is the main methyl donor of the cell and methylates DNA, RNA, proteins, and lipids [10]. Folate deficiency induces elevated homocysteine concentrations, uracil accumulation and misincorporation, DNA strand breaks, abnormal DNA and protein methylation patterns, and increased apoptosis [11-15]. However, these biological mechanisms cannot explain the protective effects of additional intake of folic acid and food folate on CL/P development. Despite the limited knowledge of the effects of folate on molecular and biological pathways, mothers-to-be and the intrauterine-developing embryo and fetus are supplemented with synthetic folic acid. It is therefore of great interest to elucidate these pathways. In order to explore the possible options for advanced research of specific pathways we performed a pilot study to identify proteomic and genomic changes in response to folate addition. Our previous (unpublished) study on protein changes in response to folate revealed the involvement of glucose metabolism, energy production, nucleocytoplasmic transport, cell cycle regulation, cytoskeleton, protein processing, and DNA transcription and translation. Regarding these findings and the essential role of folate in DNA stability, methylation, and cell death, a clear genomic response is to be expected. To get a first impression of this response, Epstein Barr virus immortalized (EBV) Blymphoblast cultures were induced with 5-methyltetrahydrofolate. The orientating nature of this approach, and the use of EBV B-lymphoblasts, clearly limits the possible observation of specific developmental functions of folate in facial primordia. However, we expect that there will be mutual consequences of folate supplementation. Therefore the goals of the present study are: (1) to identify folate responsive pathways using gene expression profiling; (2) to identify possible relationships of these differential genes with embryonic pathways involved in palate formation; (3) to compare the folate responsive genes with the earlier identified proteins.

Materials and Methods

Sample selection and culture scheme

Five Epstein Barr immortalized B-lymphoblast cultures were established from venous blood samples derived from five Dutch Caucasian children (two male, three female) with a nonsyndromic, complete, unilateral cleft lip, jaw, and palate following a standard protocol [16]. Blood samples were collected 15 months after birth, during a nationwide case control study on orofacial clefting in the Netherlands, and frozen until use [17]. For this study, early passages (<10) of the EBV immortalized B-cell cultures were used. To achieve folate depletion, B-lymphoblasts were cultured in folate-free RPMI (Gibco-BRL, Gaithersburg, MD) with 10% (v/v) dialyzed fetal calf serum (Perbio; Pierce Biotechnology, Rockford, IL) and 1% (v/v) L-glutamate, sodium-pyruvate, and penicillin/streptomycin (Gibco-BRL) for 5 days. On day 5 the natural folate metabolite, 5-methyltetrahydrofolate (5-mTHF; Sigma-Aldrich, St. Louis, MO) was added to reach the target concentration of 30 nM. Folate concentrations were measured in the medium using the Modular E170 electrochemiluminescence assay (Roche Diagnostics GmBH, Mannheim, Germany). B-lymphoblasts were harvested before (day 5) and after 5-mTHF addition on day 6. The B-lymphoblasts were pelleted and washed once with 10 mL phosphate buffered saline. Pellets were snap-frozen in liquid nitrogen and stored at

-80°C until further analysis.

RNA isolation and labelling

After thawing, total RNA was isolated using the RNeasy Midi kit (Qiagen, Valencia, CA) following the manufacturer's instructions. RNA concentrations and quality were measured with the Nanodrop ND-1000 UV-VIS spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the Agilent 2100 BioAnalyzer (Agilent, Palo Alto, CA). RNA integrity was guaranteed by only using samples with an RNA integrity number of 9.2 or higher. RNA was stored at 2808C until further use. RNA was purified and labeled for hybridization using the One Cycle Target Labeling kit (Affymetrix, Santa Clara, CA) with a starting amount of 5 lg RNA. Labeled cRNA was hybridized to Human Genome U133 Plus 2.0 gene Arrays (Affymetrix) and scanned on an Affymetrix Scanner 3000 (Affymetrix).

Quantitative Real-Time RT-PCR

Validation of microarray expression data was accomplished by quantitative real-time PCR of a selection of 10 genes, that is, ANKRD11, RBBP6, pTEN, BATF3, HSP90AA1, BCCIP, HNRPD, DPP3, TSC22D3, and MTCH1. First strand cDNA synthesis was performed using 2 lg total RNA and Superscript 2 enzyme (Gibco, Carlsbad, CA), according to a standardized protocol supplied by the vendor (protocol is available on request). Real-time PCR was performed using the SYBR Green PCR Kit (Applied Biosystems, Foster City, CA) in the Opticon 2 apparatus (MJ Research, Bio-Rad Laboratories Inc., Waltham, MA). For the PCR reaction, 5 ng cDNA of each sample was used. A melting curve analysis was performed for each reaction following each experiment to ensure the presence of a single amplified product. All PCRs were performed in duplicate. The expression level of each gene was normalized to the expression level of a reference gene, β-actin.

Data analysis

Raw expression data were normalized using the Robust Multichip Average expression summary consisting of background adjustment, quantile normalization, and summarization [18]. Unsupervised hierarchical clustering and significance analysis of microarrays (SAM) were performed with Omniviz software version 3.8 (Maynard, MA) using all probe sets. For the clustering analysis the log transformed (base 2 scale) ratio of the expression values relative to the geometrical mean of the probe set was determined. To reveal differentially expressed genes, a SAM analysis was performed using a false discovery rate of less than 1 per 100 probe sets. Additional identification of classifying probe sets was performed with the GEPAS analysis suite, http://www.gepas.org [19]. Differential genes were visualized in biological pathways with the mapping software of Ingenuity Pathway systems (Ingenuity Systems, www.ingenuity.com) and in canonical pathways with the KEGG PATHWAY database (www.genome.jp/kegg/pathway.html). For the quantitative real-time RT-PCR, Wilcoxon Signed Ranked Test was used to calculate significant differences. A p value <.05 was considered statistically significant.

Results

Folate concentrations in the medium were on average 4.4 nM (standard deviation 0.4) and 25.9 nM (standard deviation 4.0), respectively, before and after the addition of 5-mTHF. The distribution of intensities of the raw microarray data showed increased average intensities for one cell line before folate addition because of high background signal. After normalization, gene expression profiles from samples of the same cell line were highly consistent and therefore grouped together prior to clustering by folate status (Fig. 1).

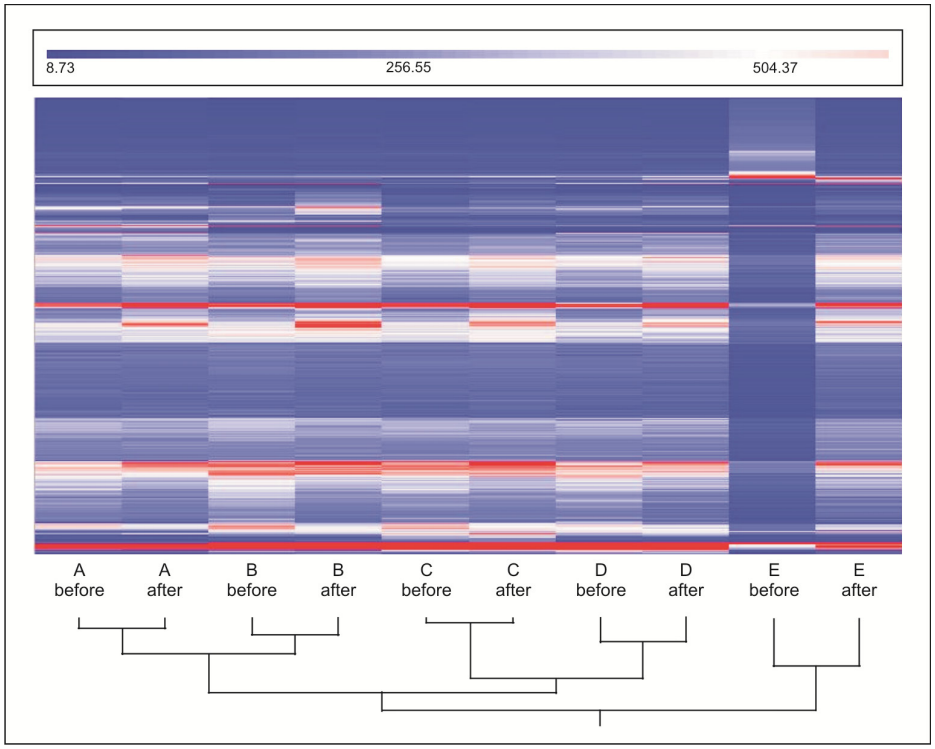
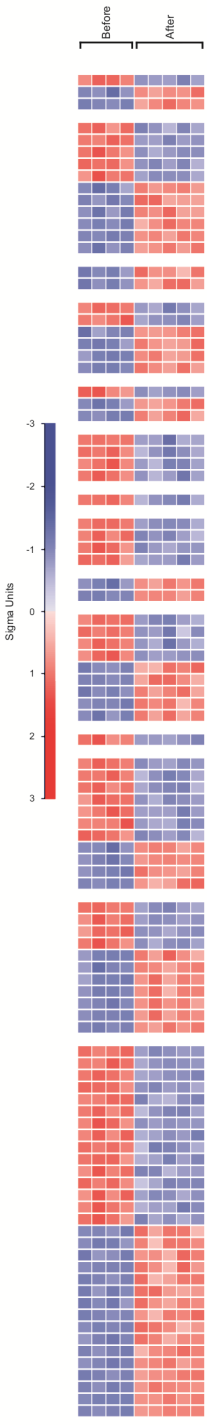


Figure 1 Unsupervised clusterplot of gene expression data including all probe sets of 5 B-lymphoblast cell lines (A–E), determined before and after 5- methyltetrahydrofolate addition. The values represent the log transformed (base 2 scale) ratio of the expression values relative to the geometrical mean of the probe sets. Though the data sets (all probe sets) were primarily clustered on originating cell line, there were also gene sets from which the expressional level seemed to alternate in correspondence with the folate status.

However, the cluster plot also showed sets of genes from which the expressional level seemed to alternate corresponding to the folate status. These potential folate-responsive genes were identified with a SAM analysis. From Figure 1 it is also clear that after normalization the ‘‘E before’’ sample, that is, the sample with the high background signals, showed very low expression levels for almost all genes. For this reason, this sample was left out of further analysis, because it did not contain any usable information. The SAM analysis was performed with the standard false discovery rate of less than 1 per 100 probe sets, corresponding to a delta-value of 4.226.



Cancer develop
- Vav 2 oncogene
- Tumor protein D52
- Zinc finger and BTB domain containing 1

Cell Cycle
- Anaphase-promoting complex 1 (meiotic checkpoint regulator)
- BRCA2 and CDKN1A interacting protein
- Nuclear distribution gene C homolog (A. nidulans)
- MCM8 minichromosome maintenance deficient 8 (S. cerevisiae)
- MCM10 minichromosome maintenance deficient 10 (S. cerevisiae)
- Phosphatase and tensine homologue (PTEN)
- Retinoblastoma binding protein 6
- Topoisomerase I binding
- Yippee-like 3 (Drosophila)
- Yippee-like 5 (Drosophila)
- Pituitary tumor-transforming 1

Cell Proliferation
- Ankyrin repeat domain 11
- Ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)

Cytoskeleton, Cell-Cell Contact
- Density-regulated protein
- Synthrophin beta 2 (SNTB2)
- Phosphatidylinositol glycan, class L
- Ras homolog gene family, member H
- Similar to Syntenin 1 (Mda-9) (Pro-TGF-alpha cytoplasmic domain-interacting protein 18) (TACIP18)
- Phosphatidylinositol glycan, class L

DNA Repair
- DNA excision repair protein ERCC-4
- Topoisomerase II alpha 170 kDa
- Single stranded DNA binding protein

Energy Metabolism
- NADH dehydrogenase (ubiquinone) Fe-S protein 6, 13kDa (NADH-coenzyme Q reductase)
- Phosphoenolpyruvate carboxykinase 2 (mitochondrial)
- ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 1
- Cytochrome c, somatic

Folate Metabolism
- Dihydrofolate reductase

HSP
- Heat shock 90kDa protein 1, alpha-like 3
- Heat shock 90kDa protein 1, alpha-like 3
- Heat shock 70kDa protein 4
- AHA1, activator of heat shock 90kDa protein ATPase homolog 1 (yeast)

Immune Respons, EBV
- CD79A antigen (immunoglobulin-associated alpha)
- CD48 antigen (B-cell membrane protein)

Protein Processing
- Dipeptidylpeptidase 3
- N-acetyltransferase 5 (ARD1 homolog, S. cerevisiae)
- AFG3 ATPase family gene 3-like 2 (yeast)
- Tubulin tyrosine ligase
- Makorin ring finger protein (MKRN1)
- V-raf murine sarcoma viral oncogene homolog B1
- V-raf murine sarcoma viral oncogene homolog B1
- Polyhomeotic like 3 (Drosophila)
- Ring finger protein 139

Signal transduction
- Nuclear receptor coactivator 5

Transcription/Translation
- Jun dimerization protein p21SNFT
- MutS homolog 6 (E. coli)
- DEAH (Asp-Glu-Ala-His) box polypeptide 9
- Isoleucine-tRNA synthetase
- Likely homolog of yeast SEN2
- Polymerase (DNA directed), alpha
- Ubiquitin-like, containing PHD and RING finger domains, 1
- Eukaryotic translation initiation factor (eIF) 2A
- Putative homeodomain transcription factor 2
- Zinc finger protein 14 (KOX 6)
- CDC-like kinase 1

Transport
- Heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37kDa)
- AP-4 complex subunit epsilon 1 (AP4E1)
- Solute carrier family 26 (sulfate transporter), member 2
- CSE1 chromosome segregation 1-like (yeast)
- KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 1
- Kinesin family member 2C
- Metallothionein 1H like protein
- Metallothionein 1A (functional)
- Metallothionein 1H
- Metallothionein 1E
- Vesicle-associated membrane protein 2 (synaptobrevin 2)

Other
- Hypothetical protein BC013949
- GT198, complete ORF
- Unknown
- KIAA1715
- KIAA0753 gene product
- KIAA1219 protein
- Hypothetical protein KIAA 1411
- SAM domain and HD domain 1
- Stimulated by retinoic acid 13
- Amyloid beta precursor protein binding protein 1, 59kDa
- Uncharacterized hypothalamus protein HT008
- Chromosome 20 open reading frame 121 (C20orf121)
- Hypothetical protein MGC10233
- CGI-12 protein
- FK506 binding protein 4, 59kDa
- Mitochondrial carrier homolog 1 (C. elegans)
- Glucocorticoid-induced leucine zipper protein
- DNA segment on chromosome X and Y (unique) 155 expressed sequence
- Unknown
- Hypothetical protein FLJ40142
- Hypothetical protein LOC401504
- Chromosome 10 open reading frame 104
- fragile histidine triad gene
- FLJ22624 protein
- Hypothetical protein 400027
- Hypothetical protein MGC2747
- Hypothetical gene CG018
- Hypothetical protein DKFZp434D2328
- Hypothetical protein LOC440836
- Adult retina protein
- Hypothetical protein FLJ32001

Figure 2 Hundred most differential genes grouped by function in B-lymphoblast cell lines before and after 5-methyltetrahydrofolate addition. The scale is presented in sigma units.

This resulted in the identification of 144 significant up-regulated and 409 down-regulated probe sets after addition of 5-mTHF. The median expression ratio was 1.63 (range: 1.2–3.8) for the significant down-regulated genes and 1.58 (range: 1.2–4.2) for the up-regulated genes. These potential folate-regulated genes are listed in Table 1 by the pathway or disease in which they are involved. These include cancer development, cell cycle checkpoint regulation, DNA replication, recombination and repair, biosynthesis of amino acids and DNA/RNA nucleotides, protein processing, and cell death. Furthermore, genes that best discriminated between pre- and postfolate-supplemented cell lines were evaluated. Figure 2 shows the 100 best classifying genes arranged by biological function. Of these classifiers, 64% were found to be significantly differentially expressed with the SAM analysis and had functions in similar pathways.

Table 1 Significant ($p < 0.0001$) folate regulated genes categorized on pathway or diseases. Certain genes were represented by multiple probe sets, which are given separately.

Gene symbol	Desciption	Mean ratio (95% CI)
Cell Cycle / Cancer		
BRCA1	breast cancer 1, early onset	0.615 (0.537 - 0.693)
CCNE2	cyclin E2	0.266 (0.216 - 0.317)
CDC7	cell division cycle 7 homolog (S. cerevisiae)	0.611 (0.529 - 0.693)
FH	fumarate hydratase	0.707 (0.667 - 0.747)
		0.669 (0.638 - 0.701)
FUS	fusion (involved in t(12;16) in malignant liposarcoma)	0.469 (0.426 - 0.512)
GART	phosphoribosylglycinamide formyltransferase	0.546 (0.471 - 0.621)
HDAC2	histone deacetylase 2	0.781 (0.728 - 0.833)
MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1	0.528 (0.448 - 0.608)
MSH3	mutS homolog 3 (E. coli)	0.785 (0.744 - 0.826)
MSH6	mutS homolog 6 (E. coli)	0.57 (0.512 - 0.628)
PHB	prohibitin	0.554 (0.485 - 0.623)
RAD51C	RAD51 homolog C (S. cerevisiae)	0.471 (0.405 - 0.536)
SVH	armadillo repeat containing 10	0.671 (0.603 - 0.738)
TUBG1	tubulin, gamma 1	0.572 (0.505 - 0.639)
ZWINT	ZW10 interactor	0.702 (0.649 - 0.755)
AURKA	aurora kinase A	1.55 (1.441 - 1.659)
CCDC28A	coiled-coil domain containing 28A	1.409 (1.307 - 1.511)
CCNB1	cyclin B1	1.564 (1.433 - 1.695)
CCNB2	cyclin B2	1.57 (1.375 - 1.765)
CCNG1	cyclin G1	1.582 (1.418 - 1.745)
CDC25C	cell division cycle 25 homolog C (S. pombe)	1.573 (1.437 - 1.709)
CDCA8	cell division cycle associated 8	1.637 (1.506 - 1.768)

CDKN3	cyclin-dependent kinase inhibitor 3	1.541 (1.256 - 1.826)
	(CDK2-associated dual specificity phosphatase)	
CSE1L	CSE1 chromosome segregation 1-like (yeast)	0.489 (0.433 - 0.546)
FHIT	fragile histidine triad gene	2.18 (1.942 - 2.418)
MDM2	Mdm2 p53 binding protein homolog (mouse)	2.218 (1.370 - 3.066)
NEK2	NIMA (never in mitosis gene a)-related kinase 2	2.135 (1.880 - 2.390)
PRDM2	PR domain containing 2, with ZNF domain	1.841 (1.600 - 2.083)
PTEN	phosphatase and tensin homolog	2.144 (1.848 - 2.441)
	(mutated in multiple advanced cancers 1)	2.101 (1.761 - 2.442)
PTTG1	pituitary tumor-transforming 1	1.592 (1.494 - 1.69)
TPX2	TPX2, microtubule-associated, homolog (Xenopus laevis)	1.609 (1.452 - 1.766)
TTC3	tetratricopeptide repeat domain 3	1.354 (1.276 - 1.432)
Cell Death		
MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1	0.528 (0.448 - 0.608)
PDCD8	apoptosis-inducing factor, mitochondrion-associated, 1	0.656 (0.609 - 0.702)
SOD1	superoxide dismutase 1, soluble	0.679 (0.610 - 0.749)
	(amyotrophic lateral sclerosis 1 (adult))	
BCL2	B-cell CLL/lymphoma 2	1.671 (1.548 - 1.795)
PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A	2.194 (1.612 - 2.777)
PTEN	phosphatase and tensin homolog	2.144 (1.848 - 2.441)
	(mutated in multiple advanced cancers 1)	2.101 (1.761 - 2.442)
VEGF	vascular endothelial growth factor A	2.129 (1.783 - 2.474)
Cellular Assembly, Organization and Proliferation		
TTL	tubulin tyrosine ligase	0.38 (0.326 - 0.433)
		0.423 (0.366 - 0.480)
TOP2A	topoisomerase (DNA) II alpha 170kDa	1.561 (1.428 - 1.695)
PAFAH1B1	platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit 45kDa	0.51 (0.428 - 0.592)
VEGF	vascular endothelial growth factor A	2.129 (1.783 - 2.474)
DNA Replication, Recombination		
BCAS2	breast carcinoma amplified sequence 2	0.686 (0.62 - 0.752)
BRCA1	breast cancer 1, early onset	0.615 (0.537 - 0.693)
CDC45L	CDC45 cell division cycle 45-like (S. cerevisiae)	0.491 (0.447 - 0.535)
CDC7	cell division cycle 7 homolog (S. cerevisiae)	0.611 (0.529 - 0.693)
DFFA	DNA fragmentation factor, 45kDa, alpha polypeptide	0.646 (0.572 - 0.720)
DHX9	DEAH (Asp-Glu-Ala-His) box polypeptide 9	0.539 (0.479 - 0.599)
EBNA1BP2	EBNA1 binding protein 2	0.422 (0.354 - 0.49)
EXO1	exonuclease 1	0.606 (0.538 - 0.673)
FEN1	flap structure-specific endonuclease 1	0.46 (0.391 - 0.529)
HELLS	helicase, lymphoid-specific	0.471 (0.403 - 0.539)

HNRPAB	heterogeneous nuclear ribonucleoprotein A/B	0.56 (0.483 - 0.636)
MCM3	minichromosome maintenance complex component 3	0.598 (0.528 - 0.669)
MCM4	minichromosome maintenance complex component 4	0.474 (0.399 - 0.549)
MCM5	minichromosome maintenance complex component 5	0.598 (0.535 - 0.661)
MCM6	minichromosome maintenance complex component 6	0.554 (0.472 - 0.636)
NAP1L1	nucleosome assembly protein 1-like 1	0.468 (0.409 - 0.527)
ORC5L	origin recognition complex, subunit 5-like (yeast)	0.482 (0.416 - 0.547)
PAXIP1	PAX interacting (with transcription-activation domain) protein 1	0.585 (0.527 - 0.643)
POLA	polymerase (DNA directed), alpha 1	0.586 (0.553 - 0.618)
POLD2	polymerase (DNA directed), delta 2, regulatory subunit 50kDa	0.665 (0.600 - 0.731)
POLD3	polymerase (DNA-directed), delta 3, accessory subunit	0.67 (0.600 - 0.740)
POLE2	polymerase (DNA directed), epsilon 2 (p59 subunit)	0.507 (0.449 - 0.565)
PRPF19	PRP19/PSO4 pre-mRNA processing factor 19 homolog	0.595 (0.531 - 0.658)
PSMC3IP	PSMC3 interacting protein	0.643 (0.578 - 0.708) 0.489 (0.385 - 0.593)
RAD51C	RAD51 homolog C (S. cerevisiae)	0.471 (0.405 - 0.536)
RFC4	replication factor C (activator 1) 4, 37kDa	0.652 (0.583 - 0.721)
RPA2	replication protein A2, 32kDa	0.692 (0.629 - 0.755)
RUVBL2	RuvB-like 2	0.751 (0.704 - 0.798) 0.763 (0.707 - 0.818)
SRPK1	SFRS protein kinase 1	0.582 (0.518 - 0.645)
CD48	CD48 molecule	1.575 (1.520 - 1.630)
CENPA	centromere protein A	1.644 (1.423 - 1.866)
HNRPA1	heterogeneous nuclear ribonucleoprotein A1	1.492 (1.342 - 1.642)
KIF2C	kinesin family member 2C	1.592 (1.493 - 1.690)
MBD1	methyl-CpG binding domain protein 1	1.517 (1.354 - 1.679)
PTTG1	pituitary tumor-transforming 1	1.592 (1.494 - 1.690)
TOP2A	topoisomerase (DNA) II alpha 170kDa	1.561 (1.428 - 1.695)
Energy Production		
NDUFB2	NADH dehydrogenase 1 beta subcomplex, 2, 8kDa	0.682 (0.623 - 0.741)
NDUFB6	NADH dehydrogenase 1 beta subcomplex, 6, 17kDa	0.709 (0.643 - 0.776)
NDUFB9	NADH dehydrogenase 1 beta subcomplex, 9, 22kDa	0.728 (0.667 - 0.789)
NDUFS3	NADH dehydrogenase Fe-S protein 3, 30kDa (NADH-coenzyme Q reductase)	0.746 (0.695 - 0.797)
NDUFS6	NADH dehydrogenase Fe-S protein 6, 13kDa (NADH-coenzyme Q reductase)	0.525 (0.487 - 0.562)
Gene Expression		
BRCA1	breast cancer 1, early onset	0.615 (0.537 - 0.693)

POLA	polymerase (DNA directed), alpha 1	0.586 (0.553 - 0.618)
POLD3	polymerase (DNA-directed), delta 3, accessory subunit	0.67 (0.600 - 0.740)
MDM2	Mdm2 p53 binding protein homolog (mouse)	2.218 (1.370 - 3.066)
RBBP6	retinoblastoma binding protein 6	1.31 (1.225 - 1.396)
ZNF42	myeloid zinc finger 1	1.304 (1.227 - 1.382)
Molecular Transport		
IPO11	importin 11	0.739 (0.68 - 0.798)
KPNB1	karyopherin (importin) beta 1	0.653 (0.614 - 0.692)
NUP205	nucleoporin 205kDa	0.812 (0.775 - 0.850)
NUP214	nucleoporin 214kDa	0.468 (0.407 - 0.529)
SLC36A1	solute carrier family 36 (proton/amino acid symporter), member 1	0.693 (0.629 - 0.757)
Nucleic Acid Metabolism		
AK2	adenylate kinase 2	0.61 (0.545 - 0.675)
NME1	protein (NM23A) expressed in non-metastatic cells	0.498 (0.435 - 0.561)
Post-Translational Modification / Protein Folding		
BAG2	BCL2-associated athanogene 2	0.642 (0.565 - 0.718)
CCT3	chaperonin containing TCP1, subunit 3 (gamma)	0.573 (0.495 - 0.65)
CCT6A	chaperonin containing TCP1, subunit 6A (zeta 1)	0.466 (0.389 - 0.544) 0.475 (0.402 - 0.548)
CCT7	chaperonin containing TCP1, subunit 7 (eta)	0.621 (0.544 - 0.699)
DNAJA1	DnaJ (Hsp40) homolog, subfamily A, member 1	0.531 (0.430 – 0.632)
HSP90AA1	heat shock protein 90kDa alpha 1 (cytosolic), class A member	0.579 (0.528 - 0.630) 0.49 (0.401 - 0.578) 0.511 (0.430 - 0.593) 0.624 (0.600 - 0.648)
HSPA8	heat shock 70kDa protein 8	0.738 (0.692 - 0.784)
HSPD1	heat shock 60kDa protein 1 (chaperonin)	0.772 (0.725 - 0.818)
HSPE1	heat shock 10kDa protein 1 (chaperonin 10)	0.417 (0.340 - 0.493)
RUVBL2	RuvB-like 2 (E. coli)	0.751 (0.704 - 0.798) 0.763 (0.707 - 0.818)
TCP1	t-complex 1	0.531 (0.467 - 0.594) 0.487 (0.370 - 0.604)
RNA Post-Translational Modification		
DDX52	DEAD (Asp-Glu-Ala-Asp) box polypeptide 52	0.569 (0.475 - 0.664)
DDX56	DEAD (Asp-Glu-Ala-Asp) box polypeptide 56	0.64 (0.568 - 0.712)
NOL5A	nucleolar protein 5A (56kDa with KKE/D repeat)	0.488 (0.426 - 0.550)
SSB	Sjogren syndrome antigen B (autoantigen La)	0.633 (0.566 - 0.699)
Small Molecule Biochemistry		
ADH5	alcohol dehydrogenase 5 (class III), chi polypeptide	0.826 (0.784 - 0.868)

CYCS	cytochrome c, somatic	0.587 (0.535 - 0.639)
DHFR	dihydrofolate reductase	0.53 (0.454 - 0.606)
		0.493 (0.445 - 0.542)
FABP5	fatty acid binding protein 5 (psoriasis-associated)	0.427 (0.347 - 0.506)
RARS	arginyl-tRNA synthetase	0.737 (0.687 - 0.788)
TSTA3	tissue specific transplantation antigen P35B	0.786 (0.739 - 0.833)
		0.708 (0.667 - 0.749)
UMPS	uridine monophosphate synthetase	0.578 (0.501 - 0.655)
UNG	uracil-DNA glycosylase	0.531 (0.468 - 0.594)
ABCB7	ATP-binding cassette, sub-family B (MDR/TAP), member 7	1.578 (1.401 - 1.754)
CD79A	CD79a molecule, immunoglobulin-associated alpha	1.426 (1.312 - 1.541)
		1.469 (1.395 - 1.542)
DHPS	deoxyhypusine synthase	1.551 (1.411 - 1.691)
DPM3	dolichyl-phosphate mannosyltransferase polypeptide 3	1.665 (1.496 - 1.834)
RHOQ	ras homolog gene family, member Q	1.769 (1.568 - 1.97)
		1.929 (1.697 - 2.161)

Projection of the data on known canonical pathways reveals involvement of various pathways in cell cycle regulation and phases of the cycle. DNA replication control is represented by regulation of various DNA polymerases and genes from the MCM family and CDC7 involved in the initiation of genome replication. The G2/ M DNA damage checkpoint was represented by the CDC25/CDC2/Cyclin B pathway, associated with DNA damage processing via the P53 tumor suppressor. The regulation of mitosis was represented with, for example, NEK2, a centriole division gene, and AURKA, which formats and stabilizes microtubules at the mitotic spindle pole during chromosome segregation. Furthermore, the CENPA gene, a methylated variant of histone H3 involved in centrosome formation, was found to be significantly up-regulated. Other pathways linked to cell cycle regulation, such as the nucleotide excision repair pathway, showed diminished activity after folate addition. This was demonstrated with decreased expression of DNA binding proteins ERCC4 and DDB1 and increased expression of single stranded DNA binding protein and various DNA polymerases. The apoptosis pathway was represented by BCL-2, FHIT, DFFA, and PDCD8. Interestingly, FHIT encompasses the FRA3B fragile site, which is expressed in a folate- deficient environment. Besides cell cycle regulation, protein processing pathways were represented by various genes of the chaperonin- containing TCP1 complex family, which were downregulated after folate addition. This complex folds various polypeptides in an ATP-dependent manner into active proteins, including actin and tubulin. Other known functions of folate, such as the one-carbon group cycle and nucleotide synthesis, were mainly unregulated, although several genes of the purine synthesis pathway were found to be down-regulated.

The results of quantitative real-time RT-PCR on 10 genes belonging to the 100 best classifying probe sets are shown in Table 2. The reference gene b-actin showed no differential expression (ratio after/before folate addition is 1.01). For 8 out of 10 tested genes the direction of regulation found with the RT-PCR

experiments was identical to the direction found with the microarrays. Moreover, from the eight directionally correct genes, five reached statistical significance, that is, RBBP6, pTEN, ANKRD11, BATF3, and HSP90AA1. The directions of the HNRPD and the TSC22D3 genes were opposite to the microarray data.

Gene	Forward primer sequence	Reverse primer sequence	Ratio after / before folate addition	P-value
Internal control				
ACTB	gcgggaaatcgtgcgtgacatt	gatggagttgaaggtagtttcgtg	1.018	0.401
Up regulated genes				
RBBP6	acagcctagaccctcagcaa	ctcctggagcgttttcactc	2.037	0.005
PTEN	accaggaccagaggaaacct	gctagcctctggatttgacg	2.591	0.005
ANKRD11	gacaaggagcccagagacag	cactgaggctctgtccttcc	1.614	0.039
MTCH1	gaccactgaggctcttttcg	cttggcgtaggtgaagaagc	1.015	0.818
TSC22D3	accagaccatgctctccatc	cagggtcttcaacagggtgt	0.914	0.589
Down regulated genes				
BATF3	agccctgaggatgatgacag	ttcagtgcctctgtcaggtg	0.511	0.005
HSP90AA1	atgaaactgcgctcctgtct	ttcttccatgcgtgatgtgt	0.515	0.005
BCCIP	atgaggagcagggaaaacct	ccagccttcagagaaaccag	0.818	0.347
DPP3	acgaggggtatgcaacagtc	gcctcgtattccagaagctg	0.914	0.347
HNRPD	gatcctaaaagggccaaagc	gttgtccatggggagctcta	1.117	0.818

Table 2 Results of the quantitative real-time RT-PCR, including primers and fold change

Discussion

In the present study we show the results of a genomewide expression analysis in B-lymphoblasts derived from CL/P patients to identify folate-responsive genes and associated pathways and their relevance in lip and palate development. The forced clustering of the data revealed significant up-regulation of 144 and down-regulation of 409 genes in response to folate. Differential expression was confirmed with quantitative RT-PCR, which showed comparable regulation in 8 out of 10 tested genes, from which five genes reached statistical significance. The regulated genes were not concentrated in specific functions or pathways, but covered several functions at a low level. This indicates a general modifying role of folate in physiological processes, which might be connected with the extensive role of folate as a one-carbon group donor. One-carbon groups are used for the synthesis of purines and pyrimidines, proteins, and the remethylation of homocysteine into methionine, the main methyl group donor of the cell. Interruption of these basal functions leads to various types of cellular and chromosomal damage, such as uracil accumulation and incorporation, abnormal protein and CpG methylation, incorrect imprinting patterns, DNA strand breaks, aneusomy, and cell death [10, 12-14, 20]. As a result, altered folate status might influence cell cycle progression. This is supported by our data showing modified expression of a relatively high number of genes involved in cell cycle regulation, especially G2/M checkpoint regulation, S-phase initiation, and regulation of mitosis. The modest number of genes regulated by folate was confirmed by the unsupervised clustering, which demonstrated that the primary clustering of samples was to the original cell line instead of folate status. The similarities between the samples from the same culture were thus larger than the similarities in folate response. One explanation

may be the low number of folate-responsive genes or the low number of samples or even different responses to the folate intervention of the separate cell lines. In our unpublished study we identified folate-responsive proteins in 30 B-lymphoblast cell lines from CL/P and control children using a new proteomic method based on peptide fingerprinting. These proteins involved glucose metabolism, energy production, nucleocytoplasmic transport, cell cycle regulation, cytoskeleton, protein processing, and DNA transcription and translation. The present results confirm the responsiveness of these pathways to folate supplementation. This was especially true with respect to various heat shock proteins and heterogeneous nuclear ribonucleoprotein. Spiegelstein et al. [21] studied gene expression in the anterior neural tube of Theiler stage 13/14 FOLBP1 knock-out mice after feeding them a folate-deficient diet and using a 5700 gene array. Biological functions identified as being regulated by folate were comparable with those identified in our study and comprised processes such as proliferation, apoptosis, transcription, and translation. Courtemanche et al. [11] performed a study using a 695 gene targeted microarray focusing on pathways involved in cellular aging and stress. Interestingly, they also identified cell cycle and DNA damage-related expression as a consequence of folate deficiency. However, their limited array size made it impossible to explore various other pathways. Developmental genes such as TGFb and MSX1 and their receptors, which are known to contribute to CL/P development [22] and thus might be target genes for folate, were not identified as folate-responsive genes. In the case of TGFß it was reported earlier that this gene is an important regulator of apoptosis in B-cell precursors [23] and thus it seems likely that the used B-lymphoblasts were expressing TGFß. The lack of differential expression of TGFß in response to folate might therefore indicate that there is no interaction. However, in theory, the possibility remains that interaction is selectively and/or temporarily present in the developing facial structures. Additional testing is needed to clarify these possible gene-specific effects of folate. An interesting observation is that the expression of genes that code for oncogenes and tumor-suppressors was altered in the present study. Although recent reports on the association of folate and the development of certain cancers are still inconclusive, there are increasing concerns that folate deficiency as well as folate excess might contribute to cancer development [8, 24-25]. The deregulating effects of folate on normal cell development as shown by our data corroborate with this hypothesis and these results might add new starting points to unravel this apparent gene-nutrient interaction. This is one of the first studies using human cell lines for genome-wide profiling of the gene expression in response to folate. We observed >500 significant changes in expression of genes involved in a variety of biological pathways. The selection of a homogeneous group of patients all with an identical nonsyndromic cleft contributed to the validity of the results. The measurements of the folate concentration in the medium of the cultures confirmed the actual folate states and thus folate deficiency and the target folate concentration. The B-lymphoblast culture model is a frequently used model for folate studies and was found to be appropriate for the assessment of folate-responsive gene expression and, as such, informative for folate-sensitive congenital malformations such as CL/P. However, we realize that other time- and tissue-specific pathways may be active during palatal development and may contribute to CL/P development. Secondly, only five cell lines derived from CL/P patients could be profiled for this explorative study. Evidently, higher numbers of cell lines will increase the reliability

of the data and prevent false-positive identifications. Inclusion of samples derived from healthy control children would also allow case or control specific gene expression relevant for increasing the understanding of CL/P development. Future studies are needed to explore the possible role of the present set of genes in the development of CL/P and other folate-sensitive congenital malformations. This may eventually lead to the further understanding of gene-environment interactions in the development of congenital malformations such as CL/P.

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CHAPTER 6

NEW APPROACH FOR THE IDENTIFICATION OF FOLATE RELATED PATHWAYS IN HUMAN EMBRYOGENESIS

Bart JB Bliek, Annelies de Klein, Theo M Luider, Jan Lindemans, Lorette Hulsman, Coşkun Güzel, Christianne JM de Groot, Régine PM Steegers-Theunissen
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Abstract

The role of natural folate intake and synthetic folic acid supplementation in the prevention of some congenital malformations is known, but on a molecular biological level poorly understood. In a first approach to identify folate-regulated pathways in human embryogenesis, tryptic digests of Epstein Barr Virus-immortalized B-lymphoblasts proteins from 6 cleft lip and/or palate patients and 2 controls were compared using matrix assisted laser desorption ionisation – time of flight (MALDI-TOF) mass spectrometry. After immortalisation, the lymphoblasts were cultured for 22 days in folate-rich, i.e. 5-methyltetrahydrofolate (5- mTHF), or folate-free medium. On day 22, 5-mTHF was added to the folate-free cultures and the profiles on day 22 and 23 were compared. After background correction for the peptide profiles of the folate-rich cultures, we found in the folate-free media several differentially expressed peptide peaks upon addition of 5-mTHF. These peptide peaks were mass annotated and matched with the MSDB human database. The results suggest some folate-regulated protein candidates as Frizzled and the Rho GTP-ases WRCH and Chp that are known in human embryogenesis. Differential folate expressed proteins in patients and controls, however, have to be further investigated.

Introduction

Sofar, the B-vitamin folate is the most promising nutrient that contributes to the prevention of congenital malformations, such as neural tube defects, cleft lip and/or palate (CLP), limb and heart malformations [1-3]. Recently, it has been demonstrated that the periconceptional use of more than 200 µg natural folate per day from food also significantly contributes to the prevention of CLP [4]. Intracellular folate serves as a source for the synthesis of the DNA bases thymidine, adenine and guanine. Another important function of the 5-methyltetrahydrofolate (5-mTHF) form of folate is the remethylation of homocysteine into methionine. The amino acid methionine is via its metabolite S-adenosylmethionine the main methyl donor in the cell. These methyl groups are also used for DNA methylation, thereby regulating the transcription and silencing of genes. A shortage of 5-mTHF is accompanied by a mild hyperhomocysteinemia, which can be treated in most cases by folic acid. Hyperhomocysteinemia has been associated with increased risks of neural tube defects, CLP, heart malformations as well as cardiovascular diseases in later life [5-8]. Increasing evidence suggests that interactions between folate, homocysteine and specific genes in early pregnancy link vascular-related congenital malformations and other reproductive failures with cardiovascular diseases in adulthood [9]. On a cellular level, folate deficiency enhances apoptosis of cultured human trophoblasts [10]. In vitro studies have demonstrated that high homocysteine concentrations increase neural crest cell motility, migration distance, increase the neural crest cell outgrowth area and decrease neural crest cell differentiation into smooth muscle cells and nerve cells [11-12]. This is in line with the in vivo study by Rosenquist et al. [13], in which very high concentrations of homocysteine administered on top of avian embryos resulted in neural tube defects, CLP, heart malformations, and ventral midline defects in a time- and concentration-dependent manner. They also observed that folic acid supplementation was only effective in preventing spina bifida and related

central nervous system malformations when it resulted in a reduction of homocysteine levels. Interestingly, Boot et al. [14] observed a decreased expression of the extracellular matrix proteins fibrillin-2 and fibronectin after homocysteine administration. Despite these findings, knowledge on molecular biological pathways involved in embryogenesis and the remodelling of the vascular system in postnatal life by folate is limited. Nowadays, the general recommendation is to use 0.4 to 0.5 mg folic acid per day during the periconceptional period. However, the most effective and safest dose of this synthetic form of folic acid may be between 0.2 and 0.3 mg [15]. Because of folic acid fortification in some countries and folic acid supplementation programmes in most continents, there is a need to investigate these issues as well as folate-gene interactions underlying the molecular biological pathways in embryogenesis. From this background, we investigated the expression of proteins in the presence or absence of 5-mTHF in Epstein Barr virus (EBV) immortalised lymphoblasts derived from CLP patients and controls.

Materials and Methods

Sample selection and culture scheme

To investigate molecular biological pathways modulated by 5-mTHF, peripheral blood B-lymphoblasts from 8 children with CLP and 3 healthy control children without congenital malformations were selected from a total panel of 96 samples of CLP and 83 samples of control children. The samples were selected on the TT genotype of the MTHFR polymorphism to control for confounding due to variations in the MTHFR genotype. These samples were collected in the Dutch nationwide non-syndromic CLP case-control triad study conducted between 1998 and 2000. The design of this study has been described in detail [16]. After blood collection, lymphoblasts were isolated using Ficoll-gradient and stored in liquid nitrogen. For the current study stable cell lines were set up. The B-lymphoblasts were immortalised with EBV as described earlier [17] and were grown at 37°C and 5% CO2 in normal RPMI medium (Gibco, Carlsbad, CA) containing 10% (v/v) fetal calf serum (Perbio, Logan, Utah) and 2000 nM folic acid. At T = 0, aliquots of these cell lines were cultured in either folate-free RPMI medium (Gibco) with 10% folate free dialyzed fetal calf serum (Perbio), or in the same folate-free medium with the daily addition of one dose of 20 nM 5-mTHF (Fig. 1). This form of folate, i.e. 5-mTHF, is unstable, UV sensitive and has a half-life of approximately one day. Therefore, the cultures with 5-mTHF were supplemented daily to maintain the target concentration of 20 nM in the medium. After 16 days of culturing, the target concentrations of 0 nM and 20 nM folate were reached. These concentrations were maintained for another 6 days until day 22. On T = 22 days 1 x 10^6 million cells per cell line in triplicate were harvested from both cultures and a single dose of 5-mTHF (20 nM) was added to the folate-free cultures (intervention). After 1 day, on T = 23 days, again 1 x 10^6 cells per cell line in triplicate were harvested. The cell pellets were washed 4 times with PBS (Gibco) to loose the proteins from medium and fetal calf serum. The cell pellets were stored at –20°C until measurement by MALDI-TOF mass spectrometry. For validation purposes, folate concentrations were routinely measured during culturing by an electrochemiluminescence assay (Roche, Basel, Switzerland) at the Clinical Chemical Laboratory of the Erasmus MC University

Medical Center in Rotterdam.

Proteomics

After thawing, the cell pellets were lyzed by ultrasound (Ultrasonic Disruptor Sonifier II Model W-250/W-450, Bransons Utrasonics, Meppel, The Netherlands) for 1 min at 60% amplitude. After boiling of the cell pellets for 5 min and cooling to room temperature, trypsin (Promega, Cat No.: V5280, Leiden, The Netherlands) was added for digestion. After one hour the digestion was stopped by the addition of 1 µl 500 mM HCl. We used C18 ZipTips to remove interfering substances from the samples as recommended by the manufacturer (Millipore, Bedford USA). To measure peptides by MALDI-TOF mass spectrometry, 0.5 µl of the sample is dissolved into 2.5 µl matrix (α-cyano-4-hydroxy-transcinnamic acid, Bruker Daltonics, Billerica, MA). Of this sample-matrix solution 0.5 µl was pipetted onto a 400 µm 384-well anchor chip MALDI-TOF plate (Bruker Daltonics, USA) and dried for 5 min. MALDI-TOF mass spectrometry analysis (BiflexIII, Ultraflex, Bruker) was performed immediately after spotting. When MALDI-TOF/TOF mass spectrometry became available, we analyzed our samples again for better resolution. Furthermore, MALDI TOF-TOF has the possibility to determine the exact amino acid sequence of a peptide peak. Thus, the protein origin can be determined more significantly.

MALDI-TOF mass spectrometry

Peptide peak profiles from the folate-free cell pellets, derived from the mass spectrometer (Fig. 2) before (T = 22 days) and after (T = 23 days) the addition of 5-mTHF, were compared and differences in peaks were annotated. The criteria for annotating a peptide peak are as follows: a) a peak is present in all triplicate samples of one cell line before intervention and not in any of the triplicate samples of that cell line after intervention or vv; b) this difference must be found in at least 2 of the 8 different cell lines. The annotated peaks in the complex peptide mixtures were normalized with the peptide profiles found in the cell pellets cultured in the folaterich medium using FlexAnalysis v2.0 software (Bruker Daltonics). Peptide peaks that were present in the cell pellets cultured in both the folate-free and folate-rich media were left out for further analyses. Differential expressed peaks in the folate-free cultured cell pellets compared to the background spectra of the folate-rich cell pellets were mass annotated. The mass list was put in the Mascot search engine (Matrix Science, www.matrixscience.com) and matched with the MSDB human database (August 2004) at a tolerance of 200 parts per million.

Results

The immortalization and establishment of stable Blymphoblast cell lines was succesfull in 6 of 8 lymphoblast samples of CLP patients and 2 of 3 lymphoblast samples of healthy persons. Fig. 1 shows the folate concentrations determined in the medium of each cell line. At T = 0 the folate concentrations did not drop immediately. It took 16 days to reach the target folate concentrations of 0 and 20 nM folate. Low concentrations of folate are known to cause cell death in vitro. In none of the cultures growth inhibition or increased apoptosis was observed by

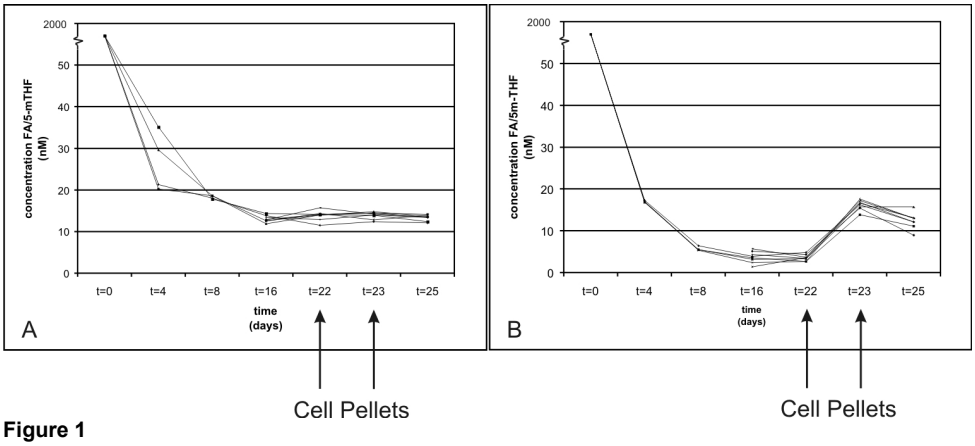

Figure 1
A Folate concentrations determined in folate-rich culture media (n = 8). Cells are harvested at days 22 and 23. B Folate concentrations determined in folate-free culture media (n = 8). At day 22, a single dose of 20 nM 5-mTHF is added to the media. Therefore, on day 23 the concentration in this group raised to 20 nM. Cells were harvested at day 22 and 23 (before and after 5-mTHF addition).

routine observation (data not shown).

The concentrations of folate in the cultures were for practical reasons maintained at 0 and 20 nM folate from T= 16 days till T= 22 days after which the cell pellets were obtained at T = 22 and T = 23 days. On day 22 the masses that correlated with the peptide peaks determined in triplicate in the folate-free cultured cell pellets before 5-mTHF administration were not significantly different from the peaks determined in triplicate in the folate-rich cultured cells (20 nM 5-mTHF).

In the comparison between day 22 and 23 within the folatefree and within the folate-rich cell pellets, fourteen peaks were present in at least two different cell lines (Fig. 2). The patient and the control group, however, were too small to identify differentially expressed peptide profiles between these two groups. Therefore, we further evaluated the peptide profiles independent of the patient or control state. By entering the 14 annotated masses in the MSDB database of Mascot (www.matrixscience.com) we found 6 known proteins and 14 hypothetical proteins in the first 20 hits of the report of identified proteins. Table 1 shows the list of these proteins. According to their ranking on the list the 6 known proteins were: Frizzled, GAP associated tyrosine phosphoprotein p62 (Sam68ΔKH), Rho family GTP-ase Chp, WRCH related GTP-ase and 3'-5' exonuclease ERI (Eri-1 homologue).

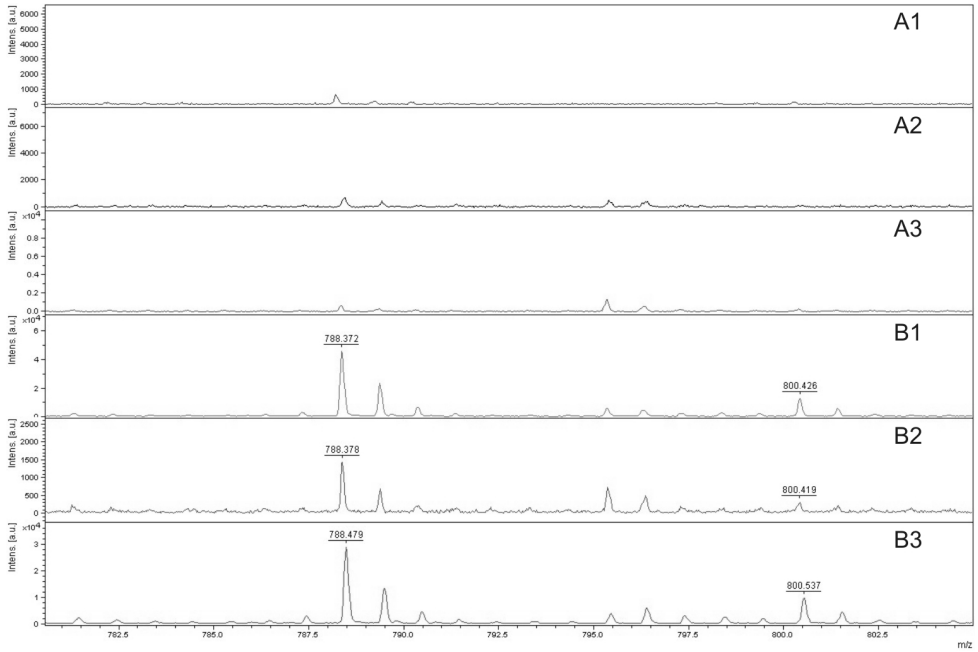

Figure 2
Figure 2 Enlargement of the peak profiles in triplicate from 1 cell line before (A1,2,3) and after (B1,2,3) administration of 5-mTHF to the folate-free cultures. The peaks with masses 788 (Frizzled, Rho-Gtp-ases WRCH and Chp) and 800 (Frizzled) were upregulated after the addition of 5-mTHF folate and maintained after correction for the peptide peaks in the folate-rich media in this cell line.

Accession	Protein mass	Score	Description
Q9BSC7	16243	40	Hypoth. protein (Fragment). (Human).
CAF14391	7933	40	AX969283 NID: - (Human).
Q9ULI4	190203	36	Hypoth. protein KIAA1236 (Fragment). (Human)
JE0338	63512	36	Frizzled-2 protein - (Human).
Q99760	44000	36	Sam68deltaKH.- (Human).
Q86UZ8	71441	35	FZD2 protein (Fragment).- (Human).
Q7Z7L8	24098	34	Hypoth. protein (Fragment).- (Human).
BAC87075	27473	34	AK127657 NID: - (Human).
AAD12740	99313	34	HSU78168 NID: - (Human).
Q8TDW6	26229	34	WRCH1-related GTPase.- (Human).
Q96L33	26201	34	Rho family GTPase Chp.- (Human).
CAE89777	25997	33	AX877551 NID: - (Human).
AAQ15128	59843	33	AF354755 NID: - (Human).
ERI1_HUMAN	39907	33	3'-5' exonuclease ERI1 homolog (Human).
CAD19332	40022	33	Sequence 1 from Patent WO0189281.- (Human).
AAQ21219	40038	33	AY310909 NID: - (Human).
Q96DP8	17781	33	Hypothetical protein FLJ30901.- (Human).
A38219	48197	32	GAP-ass. Tyr. phosphoprotein p62 – (Human).
Q9BQZ8	24076	32	DJ423B22.2 (Novel prot. similar to CE08529)
Q9GZU3	56237	32	Hypothetical protein FLJ13269.- (Human).

Table 1 The 20 proteins (MSDB database) are presented that matched with the 14 peaks after the administration of a single dose of 20 nM 5-mTHF to the folate-free lymphoblast cultures

Discussion

The EBV-immortalisation of lymphoblasts into cell lines followed by standardized culturing in folate-free RPMI medium containing 10% (v/v) dialysed fetal calf serum and 5-mTHF could be achieved in a rather uncomplicated way and is an easy model for studying the expression of proteins by folate. However, it has to be taken into account that these cells are immortalized and could therefore behave differently from the in vivo situation.

Three lymphoblast samples were not successfully immortalised, which is probably due to impaired freezing and thawing. It took a relatively long period of 16 days to reach folate depletion in the culture medium. This may be explained by the accumulation of high folate concentrations in the cells during EBV-immortalisation. During immortalisation the cells are cultured for 4-5 weeks in normal RPMI 1640 medium containing approximately 2000 nM folic acid. We assume that after switching to the folate-free medium, the relatively high number of cells (2×10^6/ml) slowly loose their stored folate to the culture medium resulting in initial high folate concentrations.

Although apoptosis is increased in human trophoblasts cultured in folate-free medium [10], the EBV-lymphoblasts cultured in folate-free media did not demonstrate elevated apoptosis rates. This finding can be explained by the relatively short time of folate-free culturing at a concentration of 0 nM 5-mTHF from day 16 to 22. Another explanation may be that immortalized lymphoblasts are relatively less sensitive to culturing in a folate-free environment.

Furthermore, proteins that are produced by the cells into the medium are not taken into account by this method, because they are washed out together with the proteins from the medium and serum.

Mass spectrometry is used for identifying single proteins from for example 2 dimensional electrophoresis gels. However, in this study, whole cell protein tryptic digests were measured. This method is very attractive because large numbers of samples can easily be measured and analyzed. Potentially the peptides that differ can be sequenced directly or after pre-fractionation. Still, standardized analysis software needs to be improved further to handle the complicated patterns that are produced by the MALDI-TOF mass spectrometer.

Some methodological issues have to be addressed. A limitation of this study is that for practical reasons it was not feasible to objectively determine cell growth and apoptosis rates by FACS and TUNEL techniques. Moreover, in this study comparisons of the peptide peaks were made after manual annotation. This is accurate but very time consuming. Therefore, we reanalyzed the data by MALDI-TOF/TOF (Ultraflex, Bruker), in which the masses are automatically identified. This data supported the peptide profiles identified by MALDI-TOF (BiflexIII, Bruker).

With the application of both methods, we found 3 of 6 upregulated known proteins on day 23 after administration of 5-mTHF to the folate-free cultures that are involved in fusion processes during human embryogenesis. The Wnt signaling pathway, from which Frizzled is a cell membrane receptor, is associated with closure of the tissues that form the palate, neural tube and heart [18-21]. Interactions between this Wnt signaling pathway and transforming growth factor beta 3 (TGFβ3), involved in the closure of the palate, have also been described before [20, 22]. The GTP-ases WRCH and Chp might also be relevant in folate-related embryogenesis, because GTP-ases are important in the polarization of

cells [23] and are described as effectors of the TGFβ3 pathway [24]. Furthermore, WRCH is reported as a possible downstream target of the Wnt signaling pathway [25]. Although these data are very interesting, they are preliminary and therefore should be considered with caution. To further validate the findings, MALDITOF/ TOF mass spectrometry should be combined with MS/MS to sequence the peptides and to confirm the initial results. Moreover, experiments with antibodies against the suggested proteins and RNA expression studies using micro-arrays and RT-PCR will substantiate the results considerably. Finally, to identify differential folatesensitivity of protein expressions between CLP patients and controls, the number of cultures should be enlarged.

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CHAPTER 7

PEPTIDE FINGERPRINTING OF FOLATE RESPONSIVE PROTEINS IN HUMAN B-LYMPHOBLASTS AND OROFACIAL CLEFTING

Bart JB Bliek, Coşkun Güzel, Annelies de Klein, Christoph Stingl, Theo M Luider, Jan Lindemans, Eric AP Steegers, Régine PM Steegers-Theunissen
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Abstract

Background Maternal periconceptional use of folic acid contributes to the prevention of neural crest related congenital malformations including orofacial clefts. The underlying biological pathways affected by folic acid, however, are still not clarified. In an explorative study, we identify folate responsive genes and pathways by advanced proteomic techniques and their possible role in orofacial development in very young children.
Materials and Methods At 15 months of age we obtained B lymphoblasts from 10 children with and 10 children without an orofacial cleft (OFC). Folate responsive protein expression was determined in folate free B-lymphoblast cultures, supplemented with 5-methyltetrahydrofolate to reach the target concentration 30nM. Folate associated differences of peptide and protein expressions were assessed by analysing samples before and folate addition. Samples were trypsin-digested and measured by nano-liquid-chromatography coupled online to a LTQ-Orbitrap mass spectrometer. Significantly differentiating peptides were searched for using a McNemar's test and correlations with proteins and existing pathways were visualized using Ingenuity Pathway Analysis.
Results We found 39 folate responsive peptides which were assigned to 30 proteins. Those proteins consisted of histones, ribosomal and heat shock proteins and proteins involved in antioxidant reactions, cytoskeleton, glycolysis and energy production, protein processing, signal transduction and translation.
Conclusions Histones, ribosomal and heat shock proteins were mainly found in the case group and here we confirm that almost 60% of these proteins were also found in a subset of the same samples in our previous study using microarray on folate responsive gene expression. The proteins were compared to known biological pathways and matched with recent relevant literature. We discuss the found proteins and pathways as a function of folate administration in a in vitro cell model.

Introduction

For more than two decades it is known that periconception intake of synthetic folic acid or natural folate reduces the birth prevalence rate of several congenital malformations, such as neural tube defects [1, 2], orofacial clefts (OFC) [3, 4] and congenital heart defects [4]. Despite the lack of knowledge on folate-gene and folate-protein interactions, this has resulted in mandatory folic acid food fortification in several countries [5] with potential side effects such as increased risk of colon cancer and asthma [6]. As one-carbon-group donor folate is essential for synthesis of the amino acids serine, cysteine and glycine, the purines adenine and guanine, the pyrimidine thymine and the remethylation of homocysteine into methionine. The methionine derivative S-adenosylmethionine is the main methyl donor for the modification of DNA, RNA, proteins and phospholipids and essential for transcriptional and (post-) translational regulation [7].
The interaction of folate on a molecular genetic level might be best understood and targeted with the methylation hypothesis, indicating a role for folate in epigenetic regulation and post-transcriptional modification [8]. Because methyl-groups are derived from the diet, in particular of folate as precursor of S-adenosylmethionine, folate is considered a modifier of gene expression [9]. Reports on the influence of folate on DNA methylation [10, 11], gene-expression [12] and DNA

hypomethylation resulting from hyperhomocysteinemia in DNA of lymphocytes [13] support this hypothesis. This is in line with evidence that the maternal nutritional state alters the epigenetic profile of the fetal genome [9, 14] and that lymphocytes can be used as a in vitro model to investigate folate uptake and response, in a general way.
In order to identify folate responsive biological pathways as a step to unravel the methylation hypothesis and its implications for orofacial clefting in human, we analysed peptide fingerprints of human B-lymphoblasts cultures before and after natural folate exposure, in children with and without OFC. The identified folate responsive protein profiles are compared to the previous microarray gene expression profiles on folate responsive gene expression in the same cell lines [15] and to relevant literature.

Materials and Methods

Sample selection

From 161 children with non-syndromic OFC and 111 control children without congenital malformations, who participated in the nationwide case-control triad study on OFC in the Netherlands between 1998 and 2003 [16], we selected 10 children with non-syndromic, complete, unilateral cleft lip, jaw and palate and 10 control children with a comparable distribution of the MTHFR 677 C>T polymorphism. The study was approved by the Central Committee for Human Research, The Hague, The Netherlands (CMO 9803-0067, 1998) and by the Medical Ethical Committees of all participating hospitals. Written informed consent was obtained from every participant and on behalf of their child before entering the study.The patient and control group consisted of 3 and 5 female samples, respectively, and 7 and 5 male samples, respectively. From the children standardized blood samples were obtained approximately 15 months after birth from which nucleated cells were isolated over a Ficoll gradient and stored in standard RPMI-medium with 10% dimethylsulfoxide in liquid nitrogen until use.

Cell culture protocol and experimental design

B-lymphoblasts were isolated and immortalised with Epstein Barr virus following a standardized protocol [17]. The 10 patient and 10 control cell lines were established and cultured until the total number of cells was around 200 million per culture (<10 passages). Whole cultures were resuspended in folate deficient medium, containing folate free RPMI 1640 (Gibco, USA), 10 % (v/v) dialyzed fetal calf serum (Perbio, USA) and 1% (v/v) 100 mM sodium-pyruvate, 200 mM L-glutamate, 10.000 units/ml penicillin and 10.000 µg/ml streptomycin (Gibco, USA) and cultured for 5 days. On day 5 the cultures were supplemented with 5-methyltetrahydrofolate (5-mTHF, Sigma-Aldrich, St. Louis, MO) to reach a target concentration of 30 nM. Before and after addition 4 million cells per culture were harvested. Folate concentrations in the medium were measured during the culturing period after appropriate dilution with standard diluent using the Modular E170 electrochemiluminescence assay (Roche Diagnostics GmBH, Germany).

Sample preparation and mass spectrometry

From each sample collection, 4 million cells per culture were harvested, pelleted and washed four times with 5 mL phosphate buffered saline. Pellets were snap-frozen in liquid nitrogen and stored at -80˚C. After thawing, cells were diluted in RapiGest^{TM} SF (Waters Corporation, Massachusetts, USA) and trypsin digested according to manufacturer's specification. From the final peptide solution 1 µL was profiled using an Ultimate 3000 nano liquid chromatography system (Dionex, Amsterdam, the Netherlands) that was coupled to a LTQ-Orbitrap (Thermo Fisher Scientific, Bremen, Germany). Settings, gradients and analysis were as described elsewhere [18]. Only peptides assigned to a protein were admitted to further analysis. Protein results were visualized with Ingenuity Pathway Analysis version 8.6 (Ingenuity Systems, Inc., Redwood City, CA).

Statistical analysis

For identification of significantly differentiating peptides the results of the 10 case and 10 control cultures were analysed as matched pairs (before versus after folate addition) using a McNemar's test, a non-parametric chi-squared statistic test with 1 degree of freedom for nominal data. For each peptide the ratio of upregulated versus downregulated pairs was statistically tested. The case and control samples were evaluated separately and grouped. A p-value smaller than 0.05 was considered significant.

RNA expression data

In our previous study we made an inventory of folate responsive genes in a subset of samples of 5 cases used in the present study [15]. RNA expression was assessed in 5 pre and 5 post folate intervention samples with Human Genome U133 Plus 2.0 gene arrays (Affymetrix). Log transformed (base 2 scale) ratios of the expression values relative to the geometrical means were calculated and submitted to a significance analysis of microarray (SAM) to obtain significantly folate regulated genes.

Results

Twenty B-lymphoblast cultures were cultured on folate free medium during 5 days to reach a folate deficient state with an average folate concentration of 4.4nM (standard deviation [SD] 0.4). Addition of the folate metabolite 5-mTHF resulted in an average concentration of 25.9nM (SD 4.0) on day 6. Subsequently, whole cell samples were trypsin digested and the peptide solutions were profiled and sequenced. This resulted in identification of on average 198 peptides per sample (SD 77) and a total of 889 unique folate responsive peptides with a peptide score of at least 25. These peptides related to 302 unique proteins.
For each of the 889 peptides presence or absence was listed per sample. For each culture the samples before and after folate addition were paired and tested with the McNemar test to compare the up regulated versus down regulated pairs. From the 889 peptides 39 peptides reached statistical significance in the case and / or control group presented in table 1 and 2. These peptides were assigned to 30 unique proteins, with a peptide distribution of 22 proteins with 1 peptide, 7 proteins

with 2 peptides and 1 protein with 3 peptides. These proteins also contained additional peptides that were not found to be significantly regulated.
The identified proteins comprised histones, ribosomal and heat shock proteins and proteins involved in antioxidant reactions, the cytoskeleton, glycolysis and energy production, protein processing, signal transduction and translation. Interestingly, this largely corresponds to the pathways found in our previous study on folate responsive RNA expression. The combined list of 30 folate regulated proteins was uploaded to Ingenuity Pathway Analysis and a theoretical network was created which was compared to known biological pathways. From the 30 identified proteins, 13 could be fitted into theoretical network related to 'cell death, cellular compromise and gene expression' (figure 1a). This network also shows an upstream relation to TGFbeta1. Projection of known canonical pathways on our set of proteins resulted in significant identification of 5 pathways for the CL/P group (figure 1b) and 12 pathways for the control group (figure 1c).
The mutual analysis of case and control groups revealed some interesting results. Especially, the 5 sequences of the heat shock proteins were all significantly regulated in the case group though not in the control group. Also the histones and ribosomal proteins were mainly found to be regulated in the case group, though not in the control group.
Our RNA expression study on folate addition only comprised CL/P samples. Therefore in the present study we compared the corresponding probe sets to the results of the CL/P group only (table 1). From the 22 case specific folate regulated proteins we found that for 11 proteins (59.1%) at least one corresponding probe set with the same direction was found significantly regulated, while the other corresponding probe sets were not significantly regulated. Two proteins showed both concordant and discordant probe sets. One protein only showed discordant probe sets. For the remaining 8 proteins there were no associated significantly regulated probe sets.

Discussion

In the last decades, the interest for underlying mechanisms of the interactions between periconceptional environmental and genetic factors in association with adverse reproductive outcome is rising. This is especially true for the B vitamin folate, which reduces the risk for congenital malformations, such as CL/P or spina bifida, when taken as supplement or via the food by mothers-to-be [4]. The underlying biological mechanisms which lead to this protective effect are thought to be the result of the role of folate as one-carbon-group (methyl) donor for regulation and programming of genes, i.e., epigenetics, and protein modifications [8]. This preliminary theory warrants exploratory studies for further support. In the present in vitro study, we made an inventory of protein pathways by means of LTQ-Orbitrap peptide fingerprinting and peptide/protein identification before and after folate addition of B-lymphoblast cell cultures from children with and without OFC. This revealed 39 significant differentiating peptides which were assigned to 30 putative folate responsive proteins consisting of histones, ribosomal and heat shock proteins and proteins involved in antioxidant reactions, the cytoskeleton, glycolysis and energy production, protein processing, signal transduction and translation.

Table 1 Significant folate responsive peptides with corresponding proteins in 10 B-lymphoblast cell cultures of CL/P patients categorized on biological function. For identification of significantly differentiating peptides the ratio of up regulated versus down regulated pairs (before and after folate addition) was statistically tested using McNemar´s test. In the penultimate two columns the corresponding probe sets from our data on folate responsive genes are shown as ratio (after/before folate addition) with 95% confidence interval. Probe sets in bold are significant in the same direction as the protein. Probe sets in italic are significant, but in the opposite direction of the protein. The last column shows references to relevant literature.

Function / Primary Amino Acid Sequence	**Protein Abbreviation**	**Protein Description**	**McNemar ratio upregulated / downregulated pairs (p-value)**	**Corresponding probe set(s)**	**Probe set ratio (95%CI)**	**Ref.[a]**
Antioxidant						
TAFQEALDAAGDK	TXN	Thioredoxin	4/0 (0.046)	**208864_s_at** **216609_at**	**0.633 (0.533-0.734)** **0.682 (0.564-0.801)**	[19]
Cytoskeleton						
VYALPEDLVEVNPK	LCP1	Plastin-2	4/0 (0.046)	208885_at	0.886 (0.732-1.041)	
YALYDATYETK	CFL1	Cofilin-1	5/0 (0.025)	**200021_at** 1555730_a_at	**0.885 (0.816-0.953)** 0.851 (0.704-0.997)	[19]
DFSALESQLQDTQELLQEENR	MYH9	Myosin-9	4/0 (0.046)	**211926_s_at**	**0.837 (0.698-0.977)**	[20-24]
Glycolysis / Energy production						
GLYGIKDDVFLSV PCILGQNGISDLVK	LDHA	L-lactate dehydrogenase A chain	4/0 (0.046)	**200650_s_at**	**0.935 (0.882-0.988)**	[25]
SLADELALVDVLEDK	LDHB	L-lactate dehydrogenase B chain	4/0 (0.046)	**201030_x_at** **213564_x_at**	**0.911 (0.867-0.954)** **0.921 (0.883-0.958)**	
SGETEDTFIADLVVGLCTGQIK	ENO1	Alpha-enolase	5/0 (0.025)	201231_s_at 217294_s_at 240258_at	1.008 (0.918-1.099) 1.121 (0.929-1.313) 1.26 (0.958-1.561)	
DPVQEAWAEDVDLR	PKM2	Pyruvate kinase isozymes M1/M2	4/0 (0.046)	201251_at	0.999 (0.885-1.113)	
GVNLPGAAVDLPAVSEK			4/0 (0.046)			
Histones						
KASGPPVSELITK	HIST1H1C	Histone H1.2	4/0 (0.046)	209398_at	1.335 (0.616-2.054)	[26-32]

KGNYAER	HIST1H2AA, HIST1H2AC, HIST2H2AA3	Histone H2A, type 1-A, Histone H2A, type 1-C, Histone H2A, type 2-A	4/0 (0.046)	215071_s_at 214290_s_at 218279_s_at 218280_x_at	1.393 (0.555-2.232) 1.408 (0.786-2.030) 0.968 (0.883-1.053) 1.24 (0.642-1.838)	
EIQTAVR	HIST1H2BB, HIST1H2BC	Histone H2B, type 1-B, Histone H2B, type 1-C/E/F/G/I	4/0 (0.046)	*214540_at* 208547_at 208490_x_at 208523_x_at 208527_x_at 214455_at 215779_s_at 236193_at	*1.234 (1.035-1.434)* 1.118 (0.626-1.610) 1.125 (0.725-1.525) 1.145 (0.627-1.662) 1.047 (0.588-1.506) 1.058 (0.686-1.430) 0.746 (0.252-1.240) 1.020 (0.648-1.392)	
Heat shock proteins						
VFIMDNCEELIPEYLNFIR	HSP90AA1	Heat shock protein HSP 90-alpha	4/0 (0.046)	**210211_s_at** **211968_s_at** **211969_at** **214328_s_at**	**0.579 (0.528-0.63)** **0.49 (0.401-0.578)** **0.511 (0.43-0.593)** **0.624 (0.6-0.648)**	[19, 33-36]
DAGTIAGLNVLR	HSPA8	Heat shock cognate 71 kDa protein	5/0 (0.025)	**208687_x_at** **210338_s_at** **221891_x_at** **224187_x_at**	**0.711 (0.638-0.785)** **0.742 (0.663-0.822)** **0.703 (0.612-0.794)** **0.738 (0.692-0.784)**	
AAVEEGIVLGGGCALLR	HSPD1	60 kDa heat shock protein	6/0 (0.014)	**200806_s_at** **200807_s_at** **243372_at** 241716_at 243845_at	**0.717 (0.602-0.832)** **0.772 (0.725-0.818)** **0.816 (0.695-0.938)** 0.952 (0.739-1.165) 0.934 (0.756-1.112)	
TALLDAAGVASLL TTAEVVVTEIPK			5/0 (0.025)			
TALLDAAGVAS LLTTAEVVVTEIPKEEK			5/0 (0.025)			
Protein production and processing						
VNPTVFFDIAVDGEPLGR	PPIA	Peptidyl-prolyl cis-trans isomerase A	4/0 (0.046)	**201293_x_at** *217602_at* 211378_x_at 211765_x_at 211978_x_at 212661_x_at 217346_at 226336_at 235741_at	**0.961 (0.937-0.985)** *1.319 (1.126-1.512)* 0.971 (0.93-1.011) 0.967 (0.929-1.006) 0.967 (0.934-1.001) 0.971 (0.928-1.013) 0.957 (0.733-1.181) 1.022 (0.903-1.141) 0.992 (0.848-1.137)	

Ribosomal proteins						
AQAAAPASVPAQAPK	RPL29	60S ribosomal protein L29	4/0 (0.046)	200823_x_at 213969_x_at 216570_x_at	1.099 (0.96-1.238) 1.07 (0.958-1.182) 1.082 (0.931-1.233)	[37-38]
LASVPAGGAVAVSAAPG SAAPAAGSAPAAAEEK	RPLP2	60S acidic ribosomal protein P2	4/0 (0.046)	**217670_at** 200908_s_at 200909_s_at	**0.773 (0.618-0.928)** 0.884 (0.695-1.072) 0.98 (0.873-1.087)	
NIEDVIAQGIGK			4/0 (0.046)			
TITLEVEPSDTIENVK	RPS27A	Ubiquitin	4/0 (0.046)	**242214_at** **244624_at** *208980_s_at* *211296_x_at* 200017_at 200633_at 217144_at	**0.539 (0.414-0.665)** **0.79 (0.648-0.932)** *1.1 (1.018-1.182)* *1.078 (1.045-1.111)* 0.988 (0.961-1.016) 0.939 (0.869-1.008) 0.888 (0.744-1.032)	
Signal transduction						
LICCDILDVLDK	YWHAE	14-3-3 protein epsilon	4/0 (0.046)	**208743_s_at** **217717_s_at** **217718_s_at** **210996_s_at** **222985_at** **212426_s_at** **213699_s_at** 200638_s_at 200639_s_at 200640_at 200641_s_at 210317_s_at 200693_at	**0.592 (0.489-0.696)** **0.634 (0.549-0.719)** **0.668 (0.61-0.727)** **0.78 (0.651-0.909)** **0.853 (0.797-0.909)** **0.75 (0.651-0.848)** **0.869 (0.82-0.918)** 1.244 (0.885-1.604) 1.12 (0.978-1.262) 1.151 (0.969-1.333) 1.364 (0.847-1.881) 1.067 (0.66-1.475) 0.965 (0.882-1.048)	
Translation						
NMITGTSQADCAVLI VAAGVGEFEAGISK	EEF1A1	Elongation factor 1-alpha 1	4/0 (0.046)	1557120_at 204892_x_at 206559_x_at 213477_x_at 213583_x_at 213614_x_at	1.102 (0.969-1.234) 0.987 (0.952-1.022) 0.982 (0.94-1.024) 0.944 (0.886-1.003) 0.961 (0.91-1.012) 0.973 (0.919-1.027)	[37, 39]

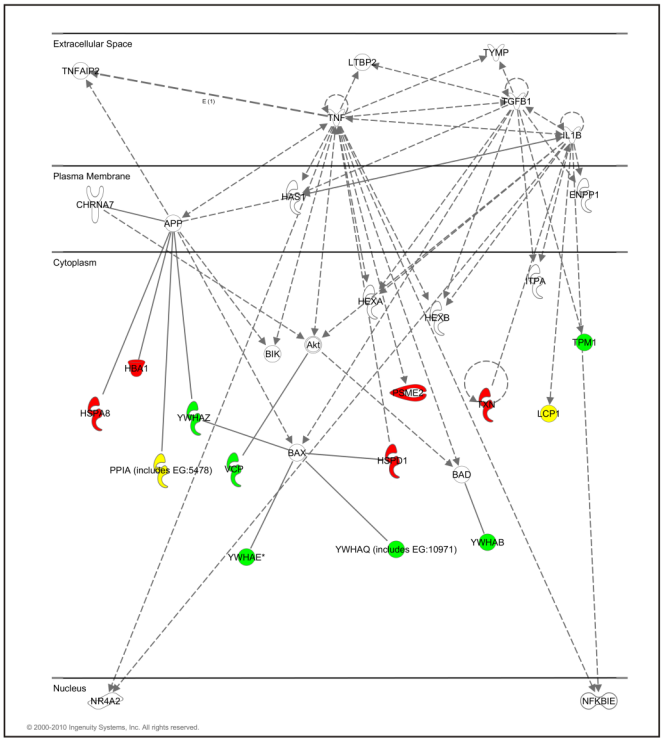
				227708_at	0.862 (0.616-1.108)	
Others						
TYFPHFDLSHGSAQVK	HBA1	Hemoglobin subunit alpha	4/0 (0.046)	204018_x_at 209458_x_at 211699_x_at 211745_x_at 214414_x_at 217414_x_at	1.038 (0.908-1.168) 1.094 (0.937-1.251) 1.083 (0.875-1.29) 1.053 (0.898-1.207) 1.056 (0.885-1.227) 1.007 (0.846-1.168)	
IIYLNQLLQEDSLNVADLTSLR	PSME2	Proteasome activator complex subunit 2	4/0 (0.046)	**201762_s_at**	**0.53 (0.478-0.583)**	

[A] References

Table 2 Significant folate responsive peptides with corresponding proteins in 10 B-lymphoblast cell cultures of healthy subjects categorized on biological function. For identification of significantly differentiating peptides the ratio of upregulated versus downregulated pairs (before and after folate addition) was statistically tested using McNemar´s test.The last column shows references to relevant literature.

Function / Primary Amino Acid Sequence	**Protein Abbreviation**	**Protein Description**	**McNemar ratio upregulated / downregulated pairs (p-value)**	**References**
Cytoskeleton				
TENLNDDEKLNNAK	LCP1	Plastin-2	0/4 (0.046)	
SSFYVNGLTLGGQK	PFN1	Profilin-1	0/5 (0.025)	
IQLVEEELDR	TPM1, TPM3	Tropomyosin alpha-1 chain, Tropomyosin alpha-3 chain	4/0 (0.046)	
Glycolysis / Energy production				
QVVESAYEVIK	LDHA	L-lactate dehydrogenase A chain	0/4 (0.046)	[25]
AAQEEYVK	ALDOA	Fructose-bisphosphate aldolase A	4/0 (0.046)	
FGANAILGVSLAVCK	ENO1	Alpha-enolase	4/0 (0.046)	

SNVSDAVAQSTR	TPI1	Triosephosphate isomerase	0/4 (0.046)	
Histones				
LAHYNKR	HIST1H2BB, HIST1H2BC	Histone H2B, type 1-B, Histone H2B, type 1-C/E/F/G/I	0/4 (0.046)	
Protein production and processing				
VNPTVFFDIAVDGEPLGR	PPIA	Peptidyl-prolyl cis-trans isomerase A	4/0 (0.046)	
NAPAIIFIDELDAIAPK	VCP	Transitional endoplasmic reticulum ATPase	0/4 (0.046)	
Ribosomal proteins				
LVILANNCPALR	RPL30	60S ribosomal protein L30	0/4 (0.046)	
TLSDYNIQK	RPS27A	Ubiquitin	0/4 (0.046)	
Signal transduction				
DSTLIMQLLR	YWHAB, YWHAE, YWHAG, YWHAQ, YWHAZ	14-3-3 protein beta, alpha, epsilon, gamma, theta, zeta/delta	5/0 (0.025)	
Translation				
EGIPALDNFLDKL	EEF2	Elongation factor 2	5/0 (0.025)	[40]
Others				
EEDDVVSEDLVQQDVQDLYEAGELK	ANXA6	Annexin A6	4/0 (0.046)	[24, 37]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APP
TPA
HEXA
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Akt
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PSMB2
HSPA8
YWHAZ
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BAX
HSPD1
PPIA (includes EG:5478)
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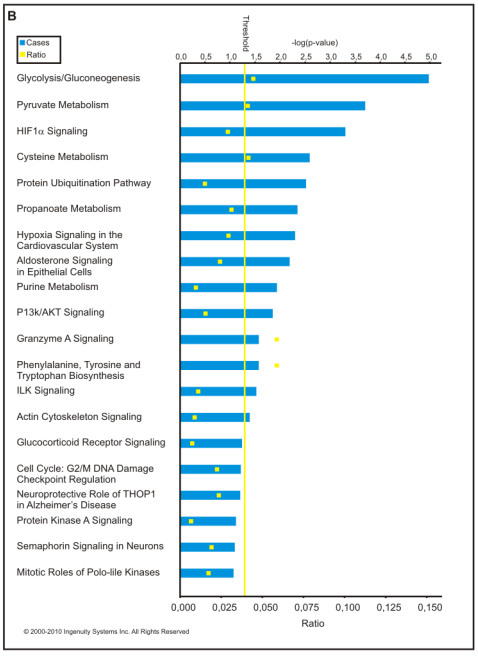
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Cases
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Pyruvate Metabolism
HIF1α Signaling
Cysteine Metabolism
Protein Ubiquitination Pathway
Propanoate Metabolism
Hypoxia Signaling in the Cardiovascular System
Aldosterone Signaling in Epithelial Cells
Purine Metabolism
P13k/AKT Signaling
Granzyme A Signaling
Phenylalanine, Tyrosine and Tryptophan Biosynthesis
ILK Signaling
Actin Cytoskeleton Signaling
Glucocorticoid Receptor Signaling
Cell Cycle: G2/M DNA Damage Checkpoint Regulation
Neuroprotective Role of THOP1 in Alzheimer's Disease
Protein Kinase A Signaling
Semaphorin Signaling in Neurons
Mitotic Roles of Polo-like Kinases
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Ratio
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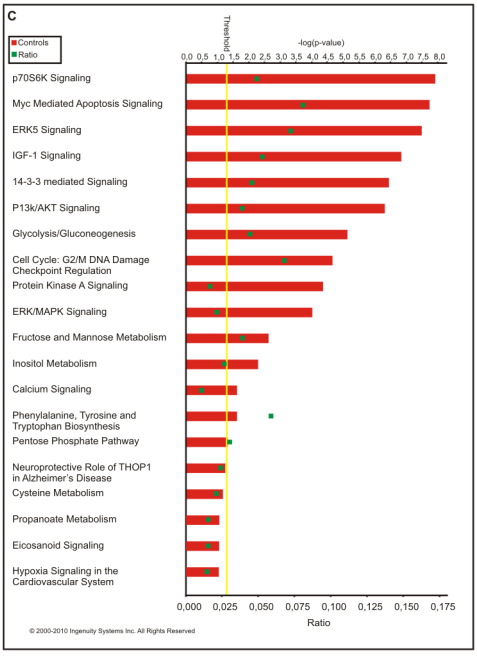
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ERK5 Signaling
IGF-1 Signaling
14-3-3 mediated Signaling
P13k/AKT Signaling
Glycolysis/Gluconeogenesis
Cell Cycle: G2/M DNA Damage Checkpoint Regulation
Protein Kinase A Signaling
ERK/MAPK Signaling
Fructose and Mannose Metabolism
Inositol Metabolism
Calcium Signaling
Phenylalanine, Tyrosine and Tryptophan Biosynthesis
Pentose Phosphate Pathway
Neuroprotective Role of THOP1 in Alzheimer's Disease
Cysteine Metabolism
Propanoate Metabolism
Eicosanoid Signaling
Hypoxia Signaling in the Cardiovascular System
0,000 0,025 0,050 0,075 0,100 0,125 0,150 0,175
Ratio
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Figure 1 Pathway analysis of folate responsive proteins
A With 13 of the 30 folate responsive proteins in B-lymphoblast cell lines of CL/P patients and controls a theoretical network could be made using Ingenuity Pathway Analysis. The proteins in red were found in case cell lines only, proteins in green were found in control cell lines only and the proteins in yellow were found in both groups. The uncolored proteins were not identified in the present study but are part of the theoretical network. **B, C** Projection of the 20 highest rated known canonical pathways on the 30 folate responsive proteins in CL/P patients (B) and controls (C). The red and blue bars represent the ratio of found proteins for a specific pathway over the total proteins in that pathway. The green and yellow dots represent the associated p-value, given as –log(p-value).

From this set a few proteins, i.e., histones, heat shock proteins and ribosomal proteins were found to be regulated only in the case and not in the control group. Literature search on these proteins revealed several direct and indirect links to folate or CL/P, but also to epigenetic mechanisms. The set of identified histones is hypothesized to be an important target for modification by methylation [19]. When assembled to nucleosomes, the histone tails are dynamically modified by acetyl, phosphor- and methyl groups which strongly influence stability and accessibility of the chromatin. Since these modifications take place after the production of the histones, this would also explain why we did not find the regulation of the histones on the RNA expression level. Association of folate with histone modification has already been demonstrated in murine prostate cell lines cultured on folate depleted and supplemented medium [20] and in liver and prostate of mice who were fed a methyl deficient diet [21, 22]. Also mutations in certain histone modifying enzymes, such as methyl transferases, are linked to a CL/P phenotype, in for example Siderius X-linked mental retardation (XLMR) syndrome (OMIM 300263) [23, 24]. These findings suggest that folate might influence gene and protein expression via chromatin modifications.
The three identified heat shock proteins (HSP), are expressed after cellular stress, which may include folate deficiency. Interestingly, down-regulation of the same HSP after folate addition was confirmed in our RNA expression study. Also other groups reported protein regulation of HSP after changing folate concentrations in liver and serum [25, 26]. Another in vitro study showed that via HSP folate protects against cellular damage of homocysteine induced stress [27]. Furthermore, interaction of HSP with the folate metabolizing enzyme dihydrofolate reductase to protect against oxidative stress is reported [28]. This is supported by a case report of elevated HSP in a woman pregnant of a CL/P child [29].
The found different ribosomal proteins which are part of the pathway of protein production and processing and comprise transport of mRNA from the nucleus to the ribosomes are indirectly involved in the transfer of methyl groups. This is concordant with our previous RNA expression data showing increased activity of translation associated genes, such as heterogeneous nuclear ribonucleoproteins, nucleopore proteins and chaperonins after folate addition. In this group, we may also regard the elongation factor 1 and 2 proteins, found in both CL/P and control group, which are necessary for translation and for which homocysteine and folate responsiveness is reported earlier [30, 31]. In this context Opitz syndrome, in which cleft palate is part of the phenotype, is of interest since mutations in the MID1 gene causing the syndrome lead to decreased association with elongation factor 1, ribonucleoproteins, nucleophosmin and annexin [32]. Also patients with Diamond-Blackfan anemia, a congenital bone-marrow-failure syndrome which is caused by mutation of ribosomal proteins, are prone to have a CL/P [33]. This data supports

the modifiable capacity of folate. Hypothetically, folate responsiveness of transcriptional and ribosomal proteins may be explained by the recent recognition of eukaryotic ribo-switches, untranslated regions of the mRNA which can bind certain metabolites, such as thiamine pyrophosphate or S-adenosylmethionine[34]. After binding, mRNA undergoes a structural change thereby inhibiting further translation. The ribo switch has been shown a downstream effector of various gene – environment interactions and some first evidence is reported that such a mechanism is present for folate[35].
A fourth set of proteins which were identified in both case and control group comprise of proteins involved in the cytoskeleton. These proteins are of interest because of their role in palatal closure, in which folate responsiveness of cofilin and myosin was reported earlier [26, 36]. Tubulin expression was decreased in an in vitro study after addition of antifolate medication[37]. But also a strong association was found for polymorphisms in the myosin-9 gene and the risk of CL/P in case-control triad studies [38-41].
Interestingly, thirteen of the folate responsive proteins could be fitted into a network and projection of known canonical pathways revealed involvement of several fundamental signaling pathways, but also in cell metabolism associated pathways. The network showed involvement of TGFbeta upstream of the folate responsive proteins. This is of special interest since TGFbeta is repeatedly associated with orofacial clefting [42] and is likely to play a role in palatal closure.
Our group previously showed that the IGF1 pathway shows association of dietary folate intake and IGF2 methylation [9]. Also the PI3/AKT is reported to be inhibited by folate [43]. Thirdly there are indication that the 14-3-3 mediated pathway which function in cell cycle checkpoint and DNA damage control is activated by homocysteine related stress in murine embryonic stem cells, an effect that is counteracted by folate [27].
Though these results are still preliminary, the fact that these folate associated proteins are found with both RNA expression and proteomic techniques and have been replicated by other groups, may lead to the conclusion that these groups of proteins / pathways are of special interest for further investigation in exploring the link between folic acid supplement use and the prevention of congenital malformation, such as CL/P.
Several methodological issues need to be addressed. To assess folate responsive proteins we used a B-lymphoblast culture model, which is a commonly used model in folate related biological studies [44-47]. We hypothesize that the proteomic folate response will be largely universal and therefore this model is very appropriate for a first explorative study. However, it is clear that for eventual extrapolation to embryonic processes other animal models are necessary to assess pathways that have a developmental dimension.
The patients and controls were randomly selected from our database to prevent selection bias though the phenotype of the clefts was identical (unilateral cheilognathopalatoschisis) and the 677C>T mutation of the MTHFR gene was equally distributed in cases and controls because of its proven effect on folate levels. The actual folate concentrations in the medium were comparable with the target concentrations and thereby further increase the validity of our results.
Though several of the identified proteins may play a role in pathways related to the folate pathway or the embryogenesis of the orofacial region, it is evident that only part of the folate responsive proteins is identified in this study. On average 198

peptides per sample (SD 77) were identified with a total of 889 unique peptides that could be measured and sequenced, indicating the complexity of the digested samples. This includes a large part of the most abundant peptides and proteins, including house-hold proteins, such as actin and glycolysis, that were found in a comparative amount in all samples. However, low abundant peptides that could not be sequenced will be left out the analysis. To improve the amount of sequenced peptides additional prefractionation (for example gel separation), prolonged chromatography, larger sample size or duplicate sample measurement can be considered. However, as a first exploration the present peptide list and identified proteins are representative and can act as a starting point for further experiments to increase the understanding of underlying biological mechanisms and protective properties of folate and folic acid supplement use.

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CHAPTER 8

IN VITRO FOLATE DEFICIENCY INDUCES ANEUSOMY OF CHROMOSOME 17 AND 21 WITHOUT A RELATION TO OROFACIAL CLEFTING IN MAN

Bart JB Bliek, Régine PM Steegers-Theunissen, Hannie Douben, Jan Lindemans, Eric AP Steegers, Annelies de Klein
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Abstract

Background: Periconception folic acid use contributes to the protection against congenital malformations, such as neural tube defects and cleft lip with or without cleft palate (CL/P). We hypothesize that low folate levels cause DNA damage, leading to chromosomal instability and aneusomy. This study investigates whether folate deficiency affects the aneusomy frequency of chromosome 17 and 21 and whether the sensitivity for aneusomy differs between CL/P patients and controls.
Methods: Epstein-Barr virus immortalized B-lymphoblasts, derived from 15 CL/P children and 15 controls, were cultured in a high and low concentration of approximately 40 nM and 5 nM 5-methyltetrahydrofolate, respectively. Fluorescent in situ hybridization was used to detect chromosomes 17 and 21 fluorescent signals.
Results: A significant increase in aneusomy of chromosomes 17, 2.3% versus 7.6% ($p \leq 0.001$), and 21, 2.5% versus 7.0% ($p \leq 0.001$) was observed after 10 days of low folate culturing. These results were comparable in patient and control cell lines. Interestingly, for chromosome 17 folate deficiency mainly resulted in an increase of monosomy (6%, $p \leq 0.001$) while for chromosome 21 an increase of trisomy was observed (4.9%, $p \leq 0.001$) was larger.
Conclusions: These data suggest that folate deficiency is an significant risk factor in the development of aneusomy and may affect the distribution of chromosomes during cell division. The comparable aneusomy frequencies in CL/P and controls suggest that other folate-related processes are involved in the pathogenesis of CL/P, and additional investigations are needed to identify the causative mechanisms.

Introduction

Clefting of the lip with or without cleft palate (CL/P) is a common malformation among newborns with a birth prevalence rate of around 15.1 per 10,000 newborns per year in the Netherlands [1]. The heterogeneous etiology of this malformation, involving both genetic and environmental factors, is still largely unknown. In the last decades extensive research has revealed that environmental influences significantly modulate CL/P risk. [2-4].This is important because this offers opportunities to develop preventive measures. In this respect, maternal pre- and periconceptional use of folic acid in tablets or in the natural form as food folate is very interesting, since additional folic acid and folate decrease CL/P risk up to 50% while a deficiency leads to an increased risk [5-8]. Because the mother is the environment of the developing child in utero, its folate supply is provided by maternal intake and metabolism. Aberrations in the metabolism of folate by the presence of maternal antiserum against the folate receptor [9] or maternal carriership of the polymorphisms MTHFR C677T [10-11] and MTHFD1 G1958A [12] partially explain why a low maternal folate status is associated with CL/P risk. Folate is an important substrate for de novo synthesis of nucleotides and proteins and for the remethylation of homocysteine into methionine. In vitro studies have shown that folate deficiency increases uracil misincorporation, single and double stranded DNA breaks and hypomethylation [13-17]. Those features of chromosomal instability led to our hypothesis that cellular folate deficiency may affect normal chromosome distribution and that the association with CL/P may be

due to a higher sensitivity for chromosomal instability in these patients than in controls. To test this hypothesis the frequency of aneusomy, as a measure for chromosomal instability, is studied in B-lymphoblast cell lines derived from very young children with and without CL/P cultured in folate deficient and folate supplemented medium.

Methods

Sample selection

In the nationwide case-control family study on orofacial clefts performed in the Netherlands between 1998 and 2003, blood samples were collected approximately at the age of 15 months from 161 children with nonsyndromic orofacial clefts and 111 control children without major congenital malformations [11]. The study was approved by the Central Committee for Human Research in The Hague, The Netherlands (CMO 9803-0067, 1998) and by the Medical Ethical Committees of all participating hospitals. Written informed consent was obtained from every parent on behalf of their child before entering the study. From this panel we selected 15 children with nonsyndromic, complete, unilateral cleft lip, jaw and palate (CL/P) and 15 control children. The patient and control group consisted of samples of eight girls and seven boys and samples of seven girls and eight boys, respectively. Nucleated cells were isolated from the blood samples over a Ficoll gradient and stored in standard RPMI-medium with 10% dimethylsulfoxide in liquid nitrogen until use.

Immortalization and Culturing

The B-lymphoblast cell lines were set up by Epstein Barr virus immortalization following a standardized protocol [18]. From each culture baseline samples were harvested (see below). The cell lines were divided into two batches of 30 cultures. One batch was depleted from folate by resuspension in a folate free medium, consisting of folate free RPMI (Gibco-BRL, USA), 10 % (v/v) dialyzed fetal calf serum (Perbio, Pierce Biotechnology, USA) and 1% (v/v) 100 mM sodium-pyruvate, 200 mM L-glutamate, 10,000 units/ml penicillin and 10,000 µg/ml streptomycin (Gibco-BRL, USA). The other batch was cultured in the same medium with a supplementation of 5-methyltetrahydrofolate (5-mTHF), the natural folate form, to reach the target concentration of 40nM. The 5-mTHF was added daily, because of its half-life of around 24 hours. The two batches were cultured under these conditions for 10 days. Folate concentrations were measured in the medium using the Modular E170 electrochemilluminescence assay (Roche Diagnostics GmBH, Germany).
Baseline (day 0) and day 10 samples were harvested from each culture and 100µl fixative (acetic acid : methanol, 1:3) was added. The cell suspensions were washed twice with fixative and stored in fixative at -20° Celsius. The lymphoblast suspensions were spotted on microscope slides, air-dried, heated for one hour at 80ºC and stored pending further analysis.

Probe Preparation

Chromosome 17 centromere clone D17Z1 [19] and the BAC clone RP11-15H6 on chromosome 21q21.3 (Bac-Pac resources, USA) were used as probe. DNA from the clones was isolated, amplified and labeled directly with Bio-16-dUTP (BioPrime kit, Invitrogen, USA).

Fluorescent in Situ Hybridization (FISH)

FISH was performed on interphase nuclei of the acetic acid/methanol fixed B-lymphoblast from day 0 and 10 according to the standard protocol [20]. The slides were washed with 2x sodium chloride/citrate (SSC) buffer and dehydrated with 70-100% ethanol washes. After addition of 5µl probe mix and denaturation at 75ºC for two minutes, the slides were hybridized by overnight incubation at 37ºC. Next day the slides were washed with 2x SSC-buffer, a 0.1x SSC, 0.1% Tween buffer and phosphate buffered saline and 4,6-diamidino-2-phenylindole (DAPI) was added to counterstain the DNA. For each hybridization, 200 nuclei were scored blindly without knowing the culture condition and patient or control status with an Axioplan 2 Imaging microscope (Zeiss, Germany) and images were captured with the ISIS (Metasystems, Germany). A second investigator randomly checked the scoring in the same way. The number of observed fluorescent signals in each interphase nuclei is given in a mean frequency of the total counted nuclei with a standard deviation (SD). These data were calculated for the pooled and separate patient and control cell lines. Statistical analysis was done using SPSS software (SPSS inc), and p-values less than or equal to 0.05 were considered statistically significant.

Results

Nuclear probe signals were counted on day 0 and 10 and the results for chromosome 17 and 21 are depicted in figure 1. The nuclear probe signals were measured for patient and control cell lines separately, but are given as a pooled average for clarity. Significant differences between patient and control measurements are mentioned separately.
One control cell line presented a total absence of chromosome 17 signals and one patient cell line displayed up to 40% of tetrasomic nuclei of chromosome 17 and 21. Both cell lines were left out the analysis. On day 10, the average folate concentrations in the medium measured 4.6nM (standard deviation [SD] 0.8nM) for the depleted cultures and 39.3nM (SD 12.1nM) for the supplemented cultures.

Chromosome 17

On day 0, before folate supplementation or depletion, disomy of chromosome 17 was found in 97.8% (SD 0.6%) of the nuclei. Monosomy was observed in 1.0% (SD 0.4%) and trisomy was seen in 0.8% (SD 0.4%) of the nuclei.
After 10 days of folate depletion the cell lines showed a significant increase in monosomy to 6.0% (SD 1.7%, $p \leq 0.000$). This was also significantly higher than the supplemented cultures (3.2% [SD 1.2%], $p \leq 0.000$).

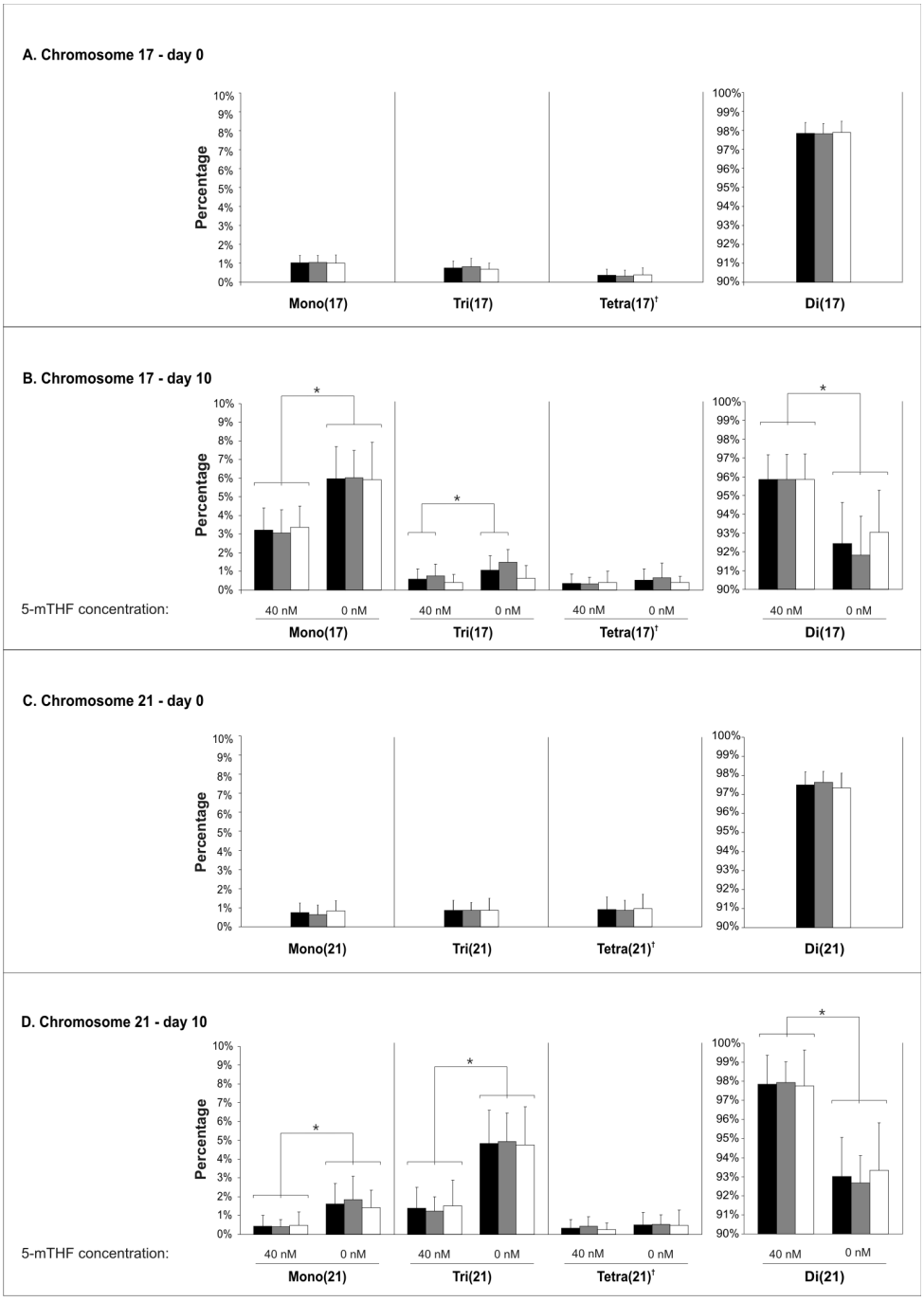
A. Chromosome 17 - day 0
Percentage
Mono(17)
Tri(17)
Tetra(17)†
Di(17)
B. Chromosome 17 - day 10
5-mTHF concentration:
40 nM
0 nM
C. Chromosome 21 - day 0
Mono(21)
Tri(21)
Tetra(21)†
Di(21)
D. Chromosome 21 - day 10

Figure 1 Percentages of mono-, di, tri- and tetrasomy of chromosome 17 and 21 at day 0 (A and C, respectively) and day 10 (B and D, respectively), subdivided into folate supplemented (40nM) and depleted (0nM) cell lines, are presented. A percentage is given for pooled patient and control cell lines (black bars), patient cell lines (grey bars) and control cell lines (white bars). Significant differences are indicated with an asterisk.

The trisomy and tetrasomy 17 frequency did not significantly differ from day 0 ($p = 0.06$ and 0.11 respectively). The frequency of trisomy 17 in folate supplemented cultures differed significantly between the patient and control group and are given separately. For the folate supplemented patient cultures the frequency of trisomy 17 was significantly lower (0.8% [SD 0.6%], $p = 0.006$) than in folate depleted patient cultures. This difference was not significant for the supplemented control cultures (0.4% [SD 0.5%], $p = 0.25$).

Chromosome 21

At day 0, before folate supplementation or depletion, disomy of chromosome 21 was found in 97.5% (SD 0.7%) of the nuclei. Monosomy 21 was observed in 0.7% (SD 0.5%) and trisomy 21 in 0.9% (SD 0.5%) of the nuclei.
After 10 days of folate depletion, monosomy 21 was increased significantly to 1.6% (SD 1.1%) compared to day 0 ($p = 0.0003$) and compared to folate supplemented cultures (0.4% [SD 0.6%], $p \leq 0.000$). The trisomy 21 frequency increased to 4.9% (SD 1.8%), which is significantly higher compared to day 0 ($p \leq 0.000$) and compared to folate supplemented cultures (1.4% [SD 1.1%], $p \leq 0.000$).

Discussion

The present study shows a significant increase of approximately 5% aneusomy of chromosomes 17 and 21 in lymphoblast cellines after 10 days culturing in a folate deficient medium, i.e., average 4.6 nM, in comparison with a folate rich culture, i.e., average 39.3 nM. This finding strongly suggests that folate deficiency can be considered a risk factor for aneusomy of chromosome 17 and 21. The increase in aneusomy together with earlier reported increases in DNA strand breaks, uracil incorporation, hypomethylation, cell growth inhibition and programmed death induced by folate deficiency, confirms the disruption of normal cell functioning by folate deficiency. Since, we did not observe differences between patient and control cultures apart from the higher frequency of trisomy 17 in depleted patient cultures, it is not very likely that these adverse effects of folate contribute to the pathogenesis of folate sensitive CL/P.
Our results confirm the increase in aneusomy in folate deficient cultured lymphoblasts reported by recent studies [21-22]. They also demonstrate that additional parameters of chromosomal instability such as micronuclei, nucleoplasmic bridges and nuclear buds can be induced in a folate deficient environment. An interesting point is that our results show a specific increase in monosomy of chromosome 17 while chromosome 21 displays more trisomy. For chromosome 17 this is in agreement with the results of the study of Wang et al. showing an approximately twofold higher frequency of monosomy 17 than trisomy 17. For chromosome 21 they found a stronger increase in monosomy instead of trisomy, though their separate measurement of cytokinesis blocked mono- and binucleated cells may have masked this difference.

We used aneusomy as an outcome measure to test for differential sensitivity for folate deficiency in CL/P patients and controls. The only significant difference was a relatively higher increase in trisomy 17 frequency of the CL/P cell lines compared to the control cell lines after 10 days of folate deficiency. Since this difference was only 0.75%, further study with prolonged culturing in a deficient medium is needed to determinate the relevance of this observation. Besides this difference the frequency of aneusomy was comparable between CL/P and control cell lines and a differential sensitivity for folate deficient aneusomy of chromosome 17 and 21 seems therefore unlikely. This data therefore suggests that the differential effects of folate in the development of the lip and palate are not reflected in pathways involved in aneusomy.

An interesting finding was the specific increment in monosomy for chromosome 17, while for chromosome 21 the increase in trisomy was the dominant effect of folate deficiency. This is possibly explained by an increased lethality of trisomy 17 and monosomy 21, but might also reflect a more chromosome specific mechanism. The increase in trisomy 21 is very exciting with regard to the recent findings and discussion on the associations of mutations in maternal folate metabolism genes and the risk of Down's syndrome in the offspring [23-28].

The reported associations between polymorphisms in folate genes such as MTHFR, MTRR, MTR and CBS and the risk of Down's syndrome are not confirmed in all study populations [29]. This can be explained by the fact that these genes are considered to be modifiers of folate availability. Together with our results we may conclude that the association with the risk of Down's syndrome may be dependent on the folate concentration of the mother, and the child as well. An exciting question to be answered is whether maternal periconception folate intake affects the risk for trisomy 21 of the child.

The evidence is clear that folate plays a role in the distribution of chromosomes [21-22]. Because of the diverse functions of folate, multiple mechanisms may however explain the results. The folate driven methylation of for example centromeric DNA and associated proteins, such as histones is thought to distinguish the centromeric region from other chromosomal regions [30]. Hypomethylation could lead to flaws during mitosis and unequal distribution of chromosomes. This hypothesis is supported by our previous studies, in which we identified the NEK2 and AURKA, which functions in centriole division and stabilization of the mitotic spindle pole, but also the CENPA histone, present in centromeric regions, as folate responsive genes [31]. This hypothesis implies that all centromeric regions would be affected by folate deficiency and would lead to aneusomic defects of every chromosome. This is in agreement with our results showing aneusomy of both chromosomes 17 and 21, though other chromosomes should be tested as well.

The presence of folate sensitive regions in the genome might also contribute to aneusomy. These fragile sites have been identified in sex and autosomal chromosomes and are expressed under folate deficient conditions [32]. Expansion of the fragile sites may lead to gaps and breaks and an unequal distribution of the chromosomes.

The strengths and weaknesses of our study need to be addressed. The present study in which we used 30 cell lines revealed the adverse effects of folate deficiency on chromosomal distribution. These results could be confirmed by comparison with pre-deficient samples and a simultaneously cultured folic acid

supplemented group. Furthermore, the folate concentrations in the medium were measured, and were found to be comparable to the target concentrations, which further validated our experiment. However, we have to consider that these lymphoblasts were EBV immortalized, which may have had a non-differential effect on the results.
In conclusion, we have demonstrated a higher frequency of monosomy 17 and trisomy 21 in a lymphoblast cell lines culture in a folate deficient environment, which was comparable in CL/P patient and control cell lines. The causative mechanisms and possible implications for the development of CL/P or other folate sensitive birth defects are a subject for further investigation.

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PART FOUR

GENERAL DISCUSSION

CHAPTER 9

GENERAL DISCUSSION

The studies described in this thesis were performed with the aim to gain new insights into gene-environment interactions, underlying pathways and mechanisms to explain the preventive effects of folic acid supplement use against in particular non-syndromic CL/P in human. This was accomplished by the execution of both epidemiological studies to investigate associations between new risk factors influenced by maternal folic acid supplement use and the occurrence of CL/P offspring (Part 1), and biological studies, in which the effects of natural folate on a chromosomal, genetic and protein level of cultured B-lymphoblasts are investigated in CL/P patients and controls (Part 2). These complementary approaches demonstrate that multidisciplinary approaches are interesting and essential to identify new folate related factors. Moreover, the understanding of molecular biological pathways in which folate is involved will lead to further development of measures to prevent non-syndromic CL/P in the future.

Part 1 Epidemiological studies

In the first part of this thesis we performed a meta-analysis to investigate associations between CL/P risk, maternal hyperhomocysteinemia, and the C677T and A1298C polymorphisms of the methylenetetrahydrofolate reductase (MTHFR) gene. Maternal hyperhomocysteinemia, a biochemical marker for a low folate status did not affect CL/P risk. Although both polymorphisms are associated with decreased expression, reduced MTHFR activity, decreased concentrations of folate and mildly elevated homocysteine concentrations [1-2], no increased CL/P risk was found for the carriers (mothers and children) of the 677T and 1298C alleles.
In the case-control study we showed that CL/P risk is increased in mothers with antiserum against the folate receptors. These results are substantiated by the lower folate and elevated homocysteine concentrations in mothers with antiserum against folate receptors. This finding is in line with studies showing the presence of this antiserum in mothers with neural tube defects in the offspring [3]. Moreover, an experimental study showed that administration of the antiserum to mice causes neural tube, cardiac and palatal malformations and embryo lethality [4].
The P-glycoprotein, encoded by the ABCB1 gene (old name MDR-1), is an efflux pump, transporting exogenous and endogenous substrates from the inside to the outside of cells. The ABCB1 3435TT genotype results in a decreased P-glycoprotein expression [5-7]. We found that the risk of CL/P offspring is increased in mothers using medication periconceptionally and carrying the ABCB1 3435TT genotype. This effect was not observed in mothers carrying the 3435CC genotype who did not use medication. A significant trend in CL/P risk was also found when the child carried the ABCB1 polymorphism and has been periconceptionally exposed to medication use of the mother. Of special interest is the first indication that periconception folic acid use may have a risk reducing effect on the interaction between medication use and the ABCB1 polymorphism of the mother.
However, before we can infer on these findings some methodological issues are addressed.

Ascertainment of CL/P

We enrolled case and control families between 1998 and 2003 at the standardized

study moment of 14 months after the birth of the index child. At the moment of inclusion the diagnosis and specification of the CL/P was carried out by specialized cleft palate teams following standardized criteria for the diagnosis of CL/P, which largely prevented misclassification of children with syndromic CL/P. Though, it always remains possible that some CL/P patients are diagnosed with a syndrome much later. However, this number will be negligible since only 5% of the cases had additional major malformations besides the CL/P which may suggest a so far undiagnosed syndrome.

Comparability of Information

Initially, control families were recruited among unrelated friends, neighbors and acquaintances of the case families and from the same population from which cases originated. However, due to the travel distance to the hospital and refusal of the ethical committees of some hospitals to recruit via case families, we had to enroll additional control families from nurseries and infant welfare centers in the surroundings of Nijmegen. This resulted in a higher educational level of the control group in comparison with the cases, which might have lead to confounding. It is suggested that a lower educational level is associated with less folic acid use and more medication use periconceptionally. However, the adjustment for education did not significantly affect the risk estimates for the investigated associations. Therefore, educational level was considered an independent risk factor for CL/P and selection bias and confounding by educational level might be very unlikely.
A strength of our study is that the homogeneity of the study groups was increased by inclusion of only Dutch Caucasian families. This prevented confounding by race, ethnicity, related lifestyle and genetic factors on the association with CL/P risk estimates and differences in distribution of the ABCB1 C3435T genotype [8]. In addition, both nonsyndromic CL/P and CP have a multifactorial origin. The risk for the CL/P phenotype, however, is presumed to be more affected by environmental influences than the CP phenotype. Therefore, we calculated the risk estimates separately for CL/P and CP, which further increased the homogeneity of the case groups.

Information bias and measurement error

The data collection by questionnaire and measurement of the folate and tHcy concentrations was collected at a fixed study moment of approximately 24 months after conception of the index-pregnancy (15 months postpartum). This study moment was chosen to limit recall bias of exposures, i.e., medication and folic acid use, in the periconception period in which also seasonal and nutritional variation are taken into account. It was shown in several studies that the nutritional intake before and after pregnancy is highly comparable [9-11]. Mothers of a child with CL/P may be more prone to admit adverse exposure to explain the CL/P in their child. On the other hand it is possible that these parents may give social desirable answers due to guilt feelings. Thus, both types of information bias lead to an under- and overestimation with a balanced effect on the risk estimates. This is confirmed in literature suggesting that recall bias might not be a big issue or has non-differential effects in case-control studies focussed on congenital malformations [12-13]. The only possibility to exclude recall bias of medication use is to check the

prescribed medication via the pharmacy records. In case of the use of over the counter medication this is not possible. A prospective cohort study or data collection after delivery would have prevented this type of confounding.
In addition, parents were considered 'medication users' if they used any type of medication during the periconception period. The used medication included corticosteroids, antibiotics, anti-epileptics, analgesics, anxiolytics, anti-psychotics, anti-depressants, antihistamines and antifungal drugs. This definition might have overestimated CL/P risk for the association between ABCB1 and medication use. Nevertheless, increased periconception exposure to medication has been accepted as a risk factor for CL/P [14] and for many drugs interactions with P-gp are unknown and have to be investigated. Future discrimination of P-gp (non)-substrates and enlarging the study populations will allow further specification of this risk.
Furthermore, at the chosen study moment the maternal metabolic and hormonal status is returned to the physiological non-pregnant situation. This resulted in the best estimates of the preconception biochemical markers.
The determination of the ABCB1 C3435T polymorphism was performed in blinded samples to prevent bias of the investigator using a standard PCR-RFLP method [15]. The genotype distributions were comparable to other Caucasian populations [5], which further strengthens the validity of this data. The auto-antibodies against the folate receptors were also determined in blinded samples with a new developed and validated method using incubation of the serum with human placental folate receptors radiolabeled with [^{3}H]folic acid [3].

Accuracy and Power

For the power calculations we used a population CL/P risk of 14.2 per 10,000 live birth and a type I error of 0.05. Maternal periconception medication use was identified as risk factor for CL/P child with a power of 73.9% (control medication use prevalence 20.5%, cases n = 175, OR = 2.2). The power of the interaction between the ABCB1 polymorphism and maternal periconception medication use was 46.0% (control medication use prevalence 20.5%, risk allele frequency 0.5, case n = 175, OR_{gene} = 1.7, $OR_{environment}$ = 2.8, $OR_{G\text{-}E}$ = 6.2). To obtain a power of ≥ 80% the sample size of both the case and control group should have been at least 130 to find medication use as risk factor for CL/P and 249 to find the gene-medication interaction. Thus, the sample size was adequate to find medication use as a the CL/P risk factor. The number of DNA samples of the case and control groups, however, were approximately half of the required sample size. This was due to the low DNA quality obtained from buccal swabs, especially in the child group, which made genotyping not possible. Furthermore, stratification of CL/P and CP phenotypes increased the homogeneity of the case groups but decreased the sample sizes. Nevertheless, despite these limitations for the first time a significant association between maternal ABCB1, periconception medication use and CL/P risk was determined. These preliminary data need confirmation in larger cohorts.
The power for the identification of maternal antiserum against folate receptors as risk factor for CL/P was 69% (control prevalence of 30%, case n = 11, OR = 10.5). If we assume that the risk estimate is between 2 and 3, which is common for other environmental factors, the sample size should have been 50 to 120 to reach a power of at least 70%.

A strength of the ABCB1 study is also the fact that a functional polymorphism was studied, e.g., ABCB1 C3435T [5, 16]. Besides the clear functional effects of the 3435TT genotype also other polymorphisms are present in the ABCB1 gene. Several studies showed that the haplotype is a better indicator for the functional consequences of this polymorphism [17]. Thus, additional genotyping of other polymorphisms should be performed to further specify the CL/P risk from the interaction between ABCB1 and medication use. Because of large racial differences in the distribution of the ABCB1 C3435T allele, investigation of other ethnic groups is needed to extrapolate these findings.
Results on the pilot study on folate receptor antiserum were validated by the concordant biochemical results thereby increasing the validity of the findings.

Part 2 Experimental studies

In the second part of this thesis a series of biological studies is presented to identify the effects of 5-methyltetrahydrofolate, the most abundant metabolite of folate, on protein and RNA levels and genomic stability. Moreover, we aimed to identify differences in these determinants and the underlying pathways between CL/P and healthy children.
The studies were performed using Epstein Barr virus immortalized B-lymphoblasts derived from CL/P and healthy children. With the rather new methodology using LTQ-Orbitrap (Thermo Fisher Scientific, Bremen, Germany) mass spectrometry connected to a liquid chromatography system, trypsin digested B-lymphoblast protein samples were profiled before and after folate addition, which resulted in the identification of 30 folate regulated proteins. These consisted of histones, ribosomal and heat shock proteins and proteins involved in antioxidant reactions, cytoskeleton, glycolysis and energy production, protein processing, signal transduction and translation.
This study was extended with RNA expression data on pre- and postintervention samples of 5 CL/P B-lymphoblast cultures to identify differential gene expression in response to 5-mTHF. In correspondence to the proteomic studies a large variety of folate responsive genes were found, which included one carbon pool and cell cycle regulation, biosynthesis of amino acids and DNA/RNA nucleotides, protein processing, apoptosis, and DNA repair. Interestingly, almost 60% of the identified proteins the corresponding gene was also found to be significantly regulated by RNA expression on folate addition.
In the third experimental study we demonstrated an increase in chromosome 17 and 21 aneusomy in CL/P and control B-lymphoblast cultures after 9 days of folate deficiency. However, the observed aneusomy was comparable for CL/P and control cell lines, indicating that genetic instability as result of low folate concentrations is not associated to failures in palate development in our B-lymphobast culture model.

Materials

For the experimental studies Epstein Barr virus immortalized B-lymphoblast cultures were used which were derived from blood samples from CL/P and healthy control children, selected from the case-control triad study that is described in the first part of the general discussion. The blood samples were obtained from the

children at an age of around 15 months. To decrease interindividual variance and to increase homogeneity among cases we selected a large group of 15 CL/P children, with a unilateral cleft of the lip, jaw and palate and 15 healthy control children who were matched on age. The MTHFR C677T genotype of the samples was chosen to resemble the general population, i.e. 55% 677CC, 45% 677CT and 5% TT, which prevented confounding by carriership of this polymorphism. For the gene-expression study the number of samples was limited to a selection of 5 cases, because of the explorative nature of the study and financial reasons.
The use of the B-lymphoblast cultures has been shown a useful model for folate related in vitro studies [18-21] because of its convenient culture properties. With Epstein Barr virus immortalization, stable cell lines were established for prolonged culturing. However, the virus might have had effects on protein or gene expression, though the chance that this has led to differential effects is probably negligible, since both case and control and pre- and post interventional samples were immortalized equally and similar passages of the cell cultures were used.
For the experimental studies, except for the gene-expression study, the data of CL/P cases and healthy controls were compared to obtain differential expressions. We assume that the differential response might also be present during embryogenesis and might therefore play a role in developmental processes. We are aware that this assumption has several limitations. We may question whether the B-lymphoblast model is appropriate for studying palatal development. It would have been ideal to use tissue samples from the developing palate in folate depleted and supplemented conditions. However, obtaining (normal) human embryonic palatal tissue samples is impossible for legal reasons and an alternative might be the use of an animal model.
It is known that certain developmental pathways are only active during embryogenesis and are not operational in postnatal life. If folate protects against CL/P via such genes those effects could not have been picked up in the present studies. It is, however, not clear whether the protecting effect of folate is mediated via these pathways or acts via general pathways that are also active in other tissues, such as in B-lymphoblasts. Our hypothesis supported by several other groups is that (dietary) folate and folic acid intake may affect the cellular methylating capacity [22-23], needed for posttranslational modification, resulting in changes in gene expression via epigenetic coding by alteration of DNA methylation patterns [24-28]. Since B-lymphoblasts effectively respond to these modifications, the B-lymphoblast culture model has been widely accepted for addressing these hypotheses. Therefore, the choice for the B-lymphoblasts model is an appropriate model to explore folate responsive expression.
The in vitro studies were performed using the 5-methyltetrahydrofolate metabolite of folate as substrate. This is a logical choice since this is the main metabolite in the blood and thus resembles the physiological situation best. Furthermore, synthetic folic acid is reduced and metabolised after uptake and eventually enters the folate cycle as the reduced 5-methyl form.

Methodology

To asses folate responsive proteins a new method was developed using mass spectrometry of trypsin digests of whole cells, also known as peptide profiling. This method has been proven successful in less complex peptide solutions, such as

digests of cerebro-spinal fluid [29-30] or serum [31]. The results of our pilot study (chapter 4) encouraged further development of the peptide profiling method. This was accomplished by the use of the newly available mass spectrometer, which is more accurate in mass determination (1-2 ppm) and by the use of additional liquid chromatography-mass spectrometry (LS-MS/MS) individual peptides can be sequenced and their protein-of-origin is accurately identified. This also excludes the potential bias in the manual analysis of the peptide profiles. The use of LS-MS/MS has several advantages. First, fractionation of the samples before mass measurement results in a much larger availability of peptides for measurement and thereby avoids the large drawback of suppression of small and low abundant peptides by large and high abundant peptides, also known as ion-suppression, in profiling unfractionated samples [31]. Secondly, LS-MS/MS is less time consuming and labour-intensive than comparable methods, such as 2D blotting. This allowed us to compare peptide profiles of 10 patient and 10 control cell lines at various time-points and in conditions of deficiency and excess of folate. Thirdly, sequencing of peptides leads to actual identification of proteins, often with multiple peptides per protein hit. Compared to the *in-silico* comparison of peptide masses used in the pilot study, this largely prevented false positive identification and led to a more thorough and reliable protein list.
However, despite these advantages, the list of 302 unique proteins should not be considered complete and still improvements can be made for the assessment of the total proteome. For example, longer and more extensive fractionation of the samples will lead to more protein hits.
To assess gene expression by RNA measurement the reliable platform of Affymetrix gene array chips was chosen and the results were analysed with validated software, resulting in a reliable list of folate responsive genes. Differential expression was confirmed with quantitative RT-PCR, which showed comparable regulation in 8 out of 10 tested genes, from which five genes reached statistical significance. Obviously, the folate responsiveness of the other listed genes needs to be confirmed with additional experiments, such as RT-PCR or immunostaining, in which the effect of different folate concentrations and time points has to be evaluated.
In the final study we assessed the development of aneusomy in response to folate depletion in human CL/P and healthy control B-lymphoblasts using fluorescent in situ hybridization. For this study the time of folate depleted culturing was longer than the previous two studies, i.e. 10 instead of 6 days. This was based on earlier reports of Courtemanche et al. and Beetstra et al. [18-19] who demonstrated biological effects of folate deficiency after 9 days. For the protein- and gene-expression studies this prolonged culturing was not obligatory since the aim was to identify expression in response to a folate intervention.
To prevent intra- and inter observer bias the counting of the slides was performed blindly and was checked by a second investigator. The control counting at the start of the experiment and the simultaneous culturing of the folate supplemented and depleted cell lines largely prevented confounding by other factors and thus the increase in aneusomy is very likely to be due to the folate deficiency. A second strength is the confirmation of aneusomy in metaphase. This proves that the interphase results are not due to artefacts and actual loss or duplication of the chromosomes is present.
The outcome measures of the four experimental studies were based on differences

in folate concentration in the culture medium. Therefore the actual concentrations in the medium were measured and further validated our results.

Inferences of the experimental findings

Our aim was to identify folate responsive pathways to explain underlying mechanisms of the role of folate in CL/P development. This was reached with both a proteomic and genomic approach. The resemblance between these three studies is depicted with a Venn diagram in figure 1. Between both proteomic studies the resulting folate responsive protein lists showed similarity in cytoskeleton and signal transduction pathways, though the proteins itself did not show resemblance. Also the signal transduction pathway concerned two different signal pathways.
This may be due to the functions of folate in protein synthesis. Folate is essential for the synthesis of the amino acids choline, serine, cysteine and glycine and for post-translational attachment of methyl-groups, indirectly derived from the folate pool via S-adenosylmethionine. We hypothesize that folate deficiency may result in a reduction of protein synthesis by substrate shortage or in abnormal protein function, transport, activity and stability, with possible increased degradation of proteins. This hypothesis is supported by several studies that reported methyl modification of proteins identified as folate responsive in our studies, such as SAM68, heterogeneous nuclear ribonucleoproteins and histones [32-34]. This is in line with our data, though further study should be performed to identify the functional consequences of these modifications. Taken together, the aspecificity of these mechanisms implicate a large diversity of affected proteins which understandably may vary between the two experiments.
Besides these biological effects, also methodological differences, as described above might have contributed to the disconcordant results. In particular, when we take into account the increase in analysable mass-range for the LTQ-Orbitrap mass spectrometer, it is likely that other more abundant peptides were found additionally. Their ion-suppressive character might have masked the peptides that were found in the first study. Secondly, the large complexity of the peptide mixtures profiled with the relatively less mass accurate BiflexIII mass spectrometry used in the first study, limited differentiation of peptides with very small mass differences. Additional fractionation, the higher accuracy and sequencing of peptides of the LTQ-Orbitrap, the distinctive capacity and thus the number of peptides was increased likewise which might have resulted in mutual differences. Therefore, independent control experiments to confirm folate responsiveness of the identified proteins should be performed, thereby validating the profiling method. In anticipation to the control experiments folate responsive gene profiling was performed. For future research using for trypsin cell digests in mass spectrometry the use of increasingly accurate mass spectrometers which are less sensitive to ion-suppression, such as the newly available Q-TOF, is crucial. Alternatively, extensive and more laborious prefractionation of all samples may prevent these drawbacks.

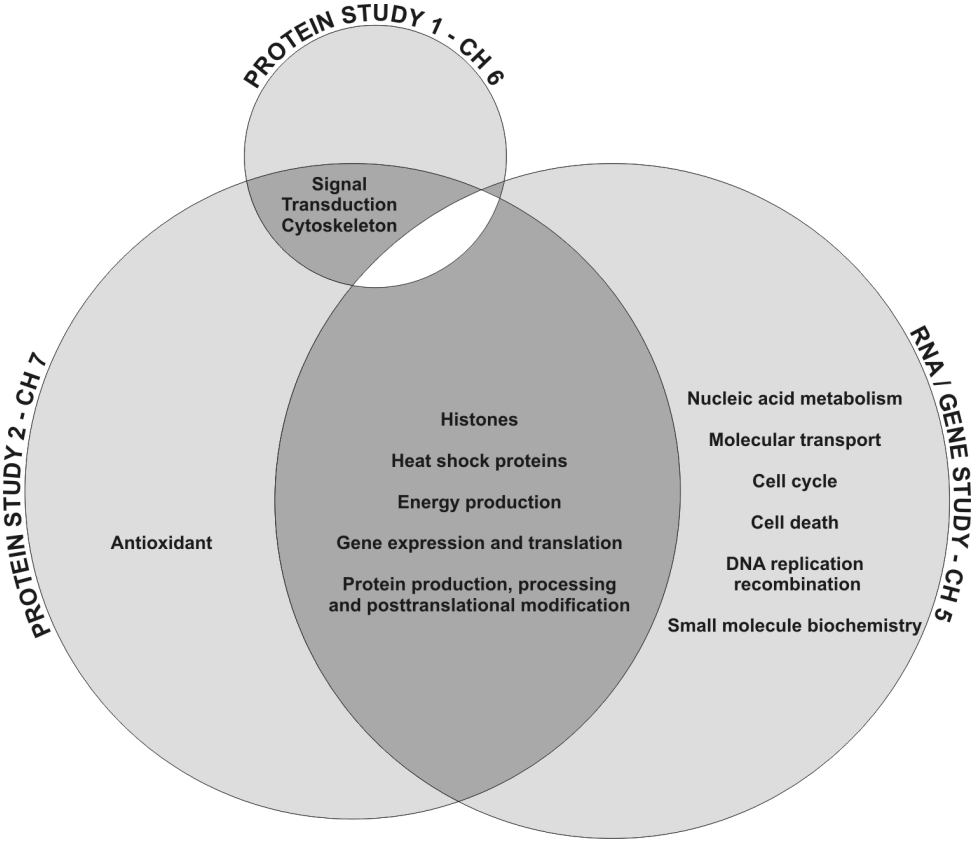

Figure 1 Venn diagram of the mutual concordance between canonical pathways from which the significant folate regulated proteins and RNA/genes of the three experimental studies originated.

Interestingly, between the second proteomic and genomic study a large agreement in folate regulated pathways was found, including histones, heat shock proteins, energy production and transcription and translation and post-translational modification. Furthermore, almost 60% of the regulated protein were also regulated on a RNA level. The first proteomic study showed no resemblance with the genomic study.

The interpretation of these results can best be targeted by the methylation pathway. Folate serves as one carbon group donor for the transition of homocysteine to methionine which in turn is the main methyldonor after activation to S-adenosylmethionine (see also Introduction). Methyl groups are used for methylation of DNA (CpG islands) and DNA associated proteins, which regulates DNA silencing and expression with various protein specific effects.

For example, the set of identified histones is an important target for modification by methylation [35]. When assembled to nucleosomes, the histone tails are dynamically modified by acetyl, phosphor- and methylgroups which strongly influence stability and accessibility of the chromatin. Earlier studies already showed that alterations in folate availability directly affect the methyl group pool and thus also the methylation state of histones [35], which may affect expressional

programming. Association of folate with histone modification has also been demonstrated in murine prostate cell lines cultured on folate depleted and supplemented medium [36] and in liver and prostate of mice who were fed a methyl deficient diet [37-38]. Also mutations in certain histone modifying enzymes, such as methyl transferases, are linked to a CL/P fenotype, in for example Siderius X-linked mental retardation (XLMR) syndrome (OMIM 300263) [39-40]. These findings indicate that folate might influence gene and protein expression via chromatin modifications.

A second interesting group are the heat shock proteins (HSP), of which three were found to be down-regulated on both protein and RNA level. The proteins are expressed after cellular stress, which may include folate deficiency. Also other groups reported protein regulation of HSP in changing folate concentrations in liver and serum [41-42]. Another in vitro study showed that via HSP folate protects against cellular damage of homocysteine induced stress [43]. Furthermore, interaction of HSP with the folate metabolizing enzyme dihydrofolate reductase to protect the latter against oxidative stress has been reported [44]. There is also a case report of elevated HSP in women pregnant of a CL/P child [45].

The third large group of interest comprise proteins involved in protein synthesis and processing. These proteins include ribosomal proteins and assistant proteins during translation. This is concordant with the RNA expression data showing increased activity of translation associated genes such as heterogeneous nuclear ribonucleoproteins, nucleopore proteins and chaperonins on folate supplementation. In this group we may also regard the elongation factor 1 and 2 proteins, found in both CL/P and control group, which are necessary for translation and for which homocysteine and folate responsiveness is reported earlier [46-47]. In the context of this group the Opitz syndrome, in which cleft palate is part of the phenotype, is of interest since mutations in the MID1 gene, causing the syndrome, lead to decreased association with elongation factor 1, ribonucleoproteins, nucleophosmin and annexin[48]. Also patients with Diamond-Blackfan anemia, a congenital bone-marrow-failure syndrome which is caused by mutation of ribosomal proteins, are prone to have a CL/P [49]. From this data it is clear that the translational activity and / or machinery is modified in response to folate. Hypothetically, folate responsiveness of these transcriptional and ribosomal proteins may be explained by the recent recognition of eukaryotic riboswitches, untranslated regions of the mRNA which can bind certain metabolites, such as thiamine pyrophosphate or S-adenosylmethionine [50]. On binding the mRNA undergoes a structural change and thereby inhibits further translation. The riboswitch has been shown a downstream effector of various gene – environment interactions and some first evidence is reported that such a mechanism is present for folate[51].

The transcriptome is probably mostly affected by epigenetic regulation via methylation of DNA and associated proteins such as histones, as is recently demonstrated for the IGF2 gene [52].

Indirectly, essential pathways needed for survival of the cell may be affected by folate deficiency, partly via earlier mentioned pathways, and may lead to cellular damage and eventually cell death. This may explain the presence of cell cycle, heat shock and glycolysis and energy production associated genes and proteins.

In contrast, the proteome might be more affected by deficient amino-acid supply and post-translational modification, leading to disturbance in protein synthesis and

increased degradation. From this point of view the aberrant translation of the folate responsive transcriptome leads to a distinctive gene and protein pattern, consistent with our results. In contrast to other vitamins such as retinoic acid, which act via specific retinoic acid receptors, we might consider folate as an indirect modifier of molecular biological mechanisms. The importance of these modifying effects in early development is an increasingly appreciated topic of research demonstrated by its role in DNA methylation and development of the lip and palate [53-54], the neural tube [55], gastro-intestinal tissues [56] and cell fate in the blastomere [57].
The abnormalities in molecular biological pathways might be a result of the increase in aneusomy frequency, as found in the final experimental study. To obtain enough time to repair the cellular and chromosomal damage the B-lymphoblast might inhibit normal cellular progression resulting in a expression of cell cycle inhibiting and repair stimulating genes and proteins. Clearly, development and cell progression is stimulated by increasing folate levels. Interestingly, recent literature described that methylation of centromeric DNA and associated histones is thought to distinct the centromeric region from other chromosomal regions [33]. Hypomethylation as a result of folate deficiency could lead to flaws during mitosis and unequal distribution of chromosomes. These interesting findings leave us with the question whether folate as indirect methyl donor influences this mechanism and ultimately causes aneuploiy. From this point of view it might even be possible that a periconception folate deficiency plays a role in increasing the risk for a trisomy 21 child causing Down syndrome. Recent studies already report on associations between mutations in maternal genes relate to folate metabolism such as MTHFR, MTRR, MTR and CBS and an increased risk for Down syndrome [58-62], though the increased risk was not confirmed in all study populations. Clearly, this subject deserves further exploration in in vitro and epidemiologic studies.
We were not able to find differences in aneusomy between CL/P and control B-lymphoblasts. The aneusomy frequency was chosen as measure for the sensitivity of damage related to folate deficiency. Apparently this approach did not differentiate between CL/P and healthy control cells. It cannot be excluded that a differential effect of folate presents during embryogenesis while the postnatal B-lymphoblasts exert a equally sensitive pattern.

Implications and conclusions

This thesis demonstrates new evidence for an increased CL/P risk by two new risk factors, being the maternal ABCB1 C3435T polymorphism combined with periconception medication use and the presence of folate receptor antiserum in the maternal blood. Identification of women with these risk factors is of importance to properly inform parents-to-be and to prevent congenital malformations if possible. Despite the evidence there is enough doubt to implement screening on the ABCB1 C3435T polymorphism and the presence of folate receptor antiserum to prevent CL/P. We can argue that the target group of medication using women is relatively small and the frequency of the homogenous mutant genotype is too low in order to screen all women. Though, the most important argument is the marginal effect on CL/P risk reduction compared to periconception folic acid use. Women are already advised to limit the use of medication and to supplement their daily folate intake with 0.4 mg of folic acid. As shown, these measures will suffice to significantly decrease CL/P risk, including mothers carrying the mutant ABCB1 C3435T

genotype. However, in future medicine when large scale genetic screening will be more common in daily practice, the determination of the ABCB1 C3435T polymorphism may be included in order to achieve a more personal risk assessments in mothers-to-be.
Thus, the current recommendation of general folic acid supplementation in the periconception period is supported by our findings. This could circumvent the adverse effects of the ABCB1 – medication interaction, but also from other polymorphisms such as the MTHFR C677T polymorphism [63]. Furthermore it could reduce the CL/P risk from folate receptor antiserum since high serum folate concentration stimulate folate transport via the reduced folate carrier [4], which remains unaffected by the antiserum.
The protective effects of folate against neural tube defects, resulted to the recommendation of periconception folic acid supplementation in a dose of 0.4-0.5mg per day in The Netherlands while other countries started folic acid enrichment of grains. Sofar, there are no clear indications that increasing folic acid intake will prevent more CL/P. Only one study of Tolarova and Harris addressed this question in a non-randomized study on the recurrence risk of CL/P using 10mg of folic acid showing comparable risk reduction as in low dose folic acid supplementation [64]. Furthermore, we should consider the possibility that high doses of synthetic folic acid may have adverse effects on other biological processes. In analogy to vitamin A, which is teratogenic in both a deficiency and excess, folic acid may be harmful in a high dose as well as in a deficient state. This is illustrated by a study of Mason et al [65] who observed an increase in the incidence of colorectal cancer after the initiation of folate enrichment of cereals. Since a causative association seems likely we should be reserved towards intake of high amounts of folic acid until we have more knowledge on the effects of folate supplementation and high serum folic acid and folate levels. As a first step in the identification of the effects of folic acid supplementation on biological processes, it revealed from the experimental studies with natural folate that a deficiency leads to a derangement of normal cell development and to aneuploidy. These effects were not observed in folate supplemented cultures indicating a beneficial and indispensable role for folate in cellular processes. Thus, also the in vitro data support an adequate intake of by mothers-to-be, and actually by all humans. However, the identified folate responsive pathways including, cell cycle, protein processing, transcription and translation, indicate that we should also be careful in increasing the recommended folate or folic acid supplementation in a total population. Also indirectly via DNA programming (methylation) we may expect that folate affects growth and development. Speculation about the harmful effects of over-stimulation via excess of folate in these basal pathways might include excessive growth and accelerated development which might even stimulate cancer [22, 66].
The present results generated new ideas for studies to clarify the interaction between folate or folic acid and several genes and proteins and the risk for CL/P with the ultimate goal of optimizing the prevention of CL/P and its tormenting impact on affected families.

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PART FIVE

ADDENDUM

Summary

Orofacial clefting (OFC) is a group of congenital malformations characterized by closure defects of the lip, jaw and/or palate. Isolated cleft of the lip and cleft lip together with cleft palate (CL/P) are considered a continuum of the same malformation and with a birth prevalence of 11.8 per 10,000, CL/P is the third most frequent congenital malformation in The Netherlands. Around 75% of CL/P occur incidentally and are not related to known syndromes. Risk factors for CL/P include both genetic mutations as well as socio-economic factors, such maternal age and educational level and life style factors such as periconceptional smoking, alcohol, medication use. Furthermore, maternal periconceptional intake of adequate amounts of folate / folic acid are related to a decreased CL/P risk comparable to the folate associated risk reduction for neural tube defects and cardial malformations. In the Netherlands these observations have led to the advise to use folic acid supplements pre- and periconceptionally, but also to mandatory folic acid food fortification in several countries. However, the underlying mechanism by which folate protects against CL/P and potential side effects of long term folic acid exposure are not clarified yet.
In this thesis we aimed to gain more insights in the role of folate and other environmental exposures and underlying mechanisms by which folate protects against CL/P. In this thesis we investigate new risk factors for CL/P by epidemiological association studies and study the in vitro effect of folate supplementation on a cellular, proteomic and genetic level.

In the first, epidemiological part we showed in a meta-analysis that two proposed risk factors for CL/P, namely maternal hyperhomocysteinemia and the C677T and A1298C polymorphism of the 5,10-methylenetetrahydrofolate reductase gene (MTHFR) in mothers and child are not independently associated with an increased CL/P risk. However, since MTHFR is important in folate metabolism, the polymorphisms may be associated with increased CL/P risk when the maternal folate status is taken into account. Such an gene-environment interaction is described in chapter 3 where we investigated the effect of maternal medication and folic acid use on the CL/P risk and the association with the C3435T polymorphism of the ABCB1 gene. This gene codes for the p-glycoprotein, a membrane protein that exports harmful substances, such as medication, out of the cell. We showed that the 2 fold increased CL/P risk associated with maternal periconceptional medication use was further increased to 6.2 when mothers have the ABCB1 3435TT genotype. This risk increased to 19.2 when mothers also did not use folic acid supplements in the periconceptional period. However, the CL/P risk associated with medication use and the ABCB1 3435TT genotype was 30% reduced when mother did use folic acid.
The cellular availability of folate does not only require adequate intake of the mother, but also adequate metabolism and transport of folates to the cell. Recent studies by da Costa et al. and Rothenberg et al. showed that autoantibodies against the folate receptor lead to a deficient folate status and cause congenital malformations such as CL/P in mice. In chapter 4 we investigated the hypothesize that mothers with these autoantibodies have an increased risk for a child with CL/P. Therefore we performed a pilot case control study in which we showed that folate receptor autoantibodies are a risk factor for CL/P since mothers with a CL/P child

significantly more often have these autoantibodies compared to mothers with a healthy child. Presence of these autoantibodies was also associated with lower serum folate and higher homocysteine levels in the mothers.
The risk factors described in this first part may be implemented in future preconceptional counselling to acquire a more personal risk profile. However, the main message of these studies is that when mothers-to-be take a sufficient amount of folate either by food or via folic acid supplements, the associated increase in CL/P risk is adequately counteracted.

In the second part of this thesis we investigated the effects of folate supplementation on RNA/gene and protein expression in B-lymphoblast cell cultures of CL/P children and healthy control children. In chapter 5 we show that folate supplementation induces significant regulation of genes involved in a variety of biological pathways, including one carbon pool and cell cycle regulation, biosynthesis of amino acids and DNA/RNA nucleotides, protein processing, apoptosis, and DNA repair. Part of these pathways were also found to be regulated on a protein level which we studied using mass spectrometry in a pilot study (chapter 6) and an extended study (chapter 7). Peptide fingerprinting was performed on trypsin digests of folate supplemented B-lymphoblasts cultures. The folate induced proteins consisted of histones, ribosomal and heat shock proteins and proteins involved in antioxidant reactions, cytoskeleton, glycolysis and energy production, translation, protein processing and signal transduction. For several of these pathways, genes and proteins, literature search showed confirmation for possible interaction with folate, as is the case for histones, heat shock proteins and proteins involved in protein synthesis and processing. The mechanism by which folate regulates these genes and proteins is hypothesized to be the methylation pathway. Folate is important as one carbon group donor for the transition of homocysteine to methionine which in turn is the main methyldonor after activation to S-adenosylmethionine (see also Introduction). Methyl groups are used for methylation of DNA (CpG islands) and DNA associated proteins, which regulates DNA silencing and expression with various protein specific effects.
In the last study (chapter 8) we show that in B-lymphoblast cultures folate deficiency is an significant risk factor for aneusomy of chromosome 17 and 21 and may affect the distribution of chromosomes during cell division. This effect is found to be equal between cultures of CL/P children and healthy children which suggests that other folate-related processes are involved in the pathogenesis of CL/P.
Taken together, folate is important in several fundamental biological pathways which may be affected in a deficient but also in (long term) supplemented state. These results indicate that we should be careful in increasing the recommended folate or folic acid supplementation in a total population. Also indirectly via DNA programming (methylation) we may expect that folate affects growth and development. Speculation about the harmful effects of over-stimulation via excess of folate in these basal pathways might include excessive growth and accelerated development which might even stimulate cancer.
With the present results new studies can be initiated to further unravel the interaction between folate or folic acid and several genes and proteins and the risk for CL/P with the ultimate goal of optimizing the prevention of CL/P and reducing its tormenting impact on affected families.

Samenvatting

Mond- en aangezichtsspleten zijn een groep aangeboren afwijkingen die gekarakteriseerd worden door sluitingsdefecten van de lip, kaak en/of het gehemelte. Een geïsoleerde lipspleet en een lipspleet in combinatie met een gehemeltespleet (CL/P) worden beschouwd als een continuüm van dezelfde afwijking en met een geboorteprevalentie van 11.8 per 10000 zijn deze de op twee na meest voorkomende aangeboren afwijking in Nederland. Ongeveer 75% van de CL/P komt solitair voor en is niet gerelateerd aan een bekend syndroom. Risicofactoren voor het krijgen van een kind met CL/P bestaan uit genetische afwijkingen als ook socio-economische factoren zoals de leeftijd en het opleidingsniveau van moeder, maar ook lifestyle factoren zoals roken en alcohol- en medicatiegebruik tijdens de conceptieperiode. Verder is ook beschreven dat voldoende inname van foliumzuur door de moeder via de voeding of via supplementen leidt tot een afname van het risico op een kind met CL/P, vergelijkbaar met de risicovermindering voor neuraalbuisdefecten (open ruggetje) en aangeboren hartafwijkingen. In Nederland hebben deze observaties geleid tot het advies voor vrouwen met kinderwens om foliumzuursupplementen te gebruiken vóór en rondom het zwanger worden, terwijl in andere landen dit zelfs geleid heeft tot verrijking van granen met foliumzuur. Toch is nog niet bekend wat de onderliggende mechanismen van het beschermende effect van foliumzuur zijn, evenals mogelijke bijwerkingen van foliumzuurblootstelling op de lange termijn. Dit proefschrift heeft tot doel het verkrijgen van meer inzicht in de rol van foliumzuur en andere omgevingsfactoren en onderliggende mechanismen van het beschermende effect van foliumzuur. In dit proefschrift onderzoeken we nieuwe risicofactoren voor CL/P met epidemiologische associatiestudies en bestuderen we met behulp van celkweken de effecten van foliumzuur op cel-, eiwit en genetisch niveau.

In het eerste, epidemiologische deel tonen we met een meta-analyse aan dat een tweetal potentiële risicofactoren voor CL/P, namelijk maternale hyperhomocysteïnemie en de C677T en A1298C polymorfismes van het 5,10-methylenetetrahydrofolate reductase gen (MTHFR) in moeders en kinderen geen onafhankelijke risicofactoren zijn voor CL/P. Echter, aangezien MTHFR belangrijk is in het metabolisme van foliumzuur, is het mogelijk dat deze polymorfismes wel een effect op het CL/P-risico hebben als de foliumzuurstatus van moeder betrokken wordt in de berekening. Een vergelijkbare gen-omgevingsinteractie wordt beschreven in hoofdstuk 3, waar we het effect van medicatie- en foliumzuurgebruik door de moeder en de associatie met het C3435T polymorfisme van het ABCB1 gen op het CL/P-risico onderzoeken. Dit gen codeert voor het p-glycoproteine, een membraaneiwit dat zorgt voor de export van verschillende schadelijke stoffen, zoals medicatie, uit de cel. We laten zien dat het risico op CL/P, dat 2 maal verhoogd is door medicatiegebruik van de moeder, verder stijgt naar 6.2 wanneer de moeder het ABCB1 3435TT genotype heeft. Wanneer de moeder ook geen foliumzuur gebruikt, stijgt dit risico verder naar 19.2. Echter, wanneer de moeder wel foliumzuur gebruikt, dan daalt het risico met 30%.
De beschikbaarheid van foliumzuur voor de cel wordt niet alleen bepaald door adequate inname door moeder, maar ook door een toereikende verwerking en transport van foliumzuur naar de cel. Recente studies van da Costa en anderen en

Rothenberg en anderen toonden aan dat autoantilichamen tegen de foliumzuurreceptor leiden tot een deficiënte foliumzuurstatus en bij muizen aangeboren afwijkingen zoals CL/P veroorzaken. In hoofdstuk 4 onderzoeken we de hypothese dat moeders met deze antilichamen een verhoogd risico op een kind met CL/P hebben. Daarom voerden we een verkennende case control studie uit, waarin we aantoonden dat autoantilichamen tegen de foliumzuurreceptor vaker voorkomen bij moeders met een kind met CL/P dan bij moeders met een gezond kind. De aanwezigheid van deze antilichamen was ook gerelateerd aan een lagere foliumzuurconcentratie en een hogere homocysteïneconcentratie in het bloed.
De risicofactoren die beschreven worden in het eerste deel van dit proefschrift kunnen in de toekomst geïmplementeerd worden in de advisering van vrouwen die zwanger willen worden, waarbij een meer persoonlijk risicoprofiel bepaald kan worden. De belangrijkste boodschap is echter dat wanneer een aanstaande zwangere een adequate hoeveelheid foliumzuur via voeding of supplementen inneemt, de beschreven risico's op CL/P afdoende afgedekt worden.

In het tweede gedeelte van dit proefschrift onderzoeken we de effecten van foliumzuur op RNA/gen- en eiwitniveau in B-lymfoblast celkweken van kinderen met CL/P en gezonde kinderen. In hoofdstuk 5 tonen we aan dat foliumzuurtoevoeging een significant effect heeft op genexpressie. De geïnduceerde genen omvatten verschillende biologische pathways, waaronder one carbon pool and cel cyclus regulatie, biosynthese van aminozuren en DNA/RNA-nucleotiden, eiwitprocessing, apoptose en DNA-herstel. Een deel van deze pathways bleek ook gereguleerd te worden op eiwitniveau, hetgeen we bestudeerden met massaspectrometrie in een oriënterende studie (hoofdstuk 6) en in een uitbreiding daarvan (hoofdstuk 7). Peptide-fingerprinting werd verricht op trypsinedigesten van foliumzuur gesupplementeerde B-lymfoblastcelkweken. De door foliumzuur gereguleerde eiwitten bestonden uit histonen, ribosomale en heat shock eiwitten en eiwitten betrokken in antioxidantreacties, het cytoskelet, glycolyse en energieproductie, translatie, eiwitprocessing en signaaltransductie. Literatuuronderzoek naar sommige van deze pathways bevestigde een mogelijke interactie tussen foliumzuur en eiwitten zoals het geval was bij de histonen, heat shock eiwitten en eiwitten betrokken in eiwitsynthese en -processing. Het mechanisme waarmee foliumzuur deze genen en eiwitten reguleert is mogelijk de methylatiepathway. Foliumzuur is belangrijk als one carbon group donor in de omzetting van homocysteine in methionine, dat weer na omzetting in S-adenosylmethionine de belangrijkste methyldonor van de cel is (zie ook introductie). Methylgroepen worden gebruikt voor methylatie van DNA (CpG eilanden) en DNA geassocieerde eiwitten, welke DNA-expressie reguleren met verscheidene eiwitspecifieke effecten.
In de laatste studie (hoofdstuk 8) tonen we aan dat foliumzuurtekort leidt tot een toename in aneusomie van chromosoom 17 en 21 in B-lymfoblastcelkweken en mogelijk de chromosoomdistributie tijdens de celdeling beïnvloedt. Deze toename werd in gelijke mate gezien in kweken van kinderen met CL/P als in gezonde kinderen, hetgeen suggereert dat andere foliumzuur gerelateerde processen betrokken zijn in de pathogenese van CL/P.
Concluderend is foliumzuur belangrijk voor verschillende basale biologische pathways, die zowel bij foliumzuurdeficiëntie als bij gesupplementeerde status op lange termijn mogelijk beïnvloed worden. Deze resultaten tonen aan dat we

terughoudend moeten zijn met het verhogen van de aanbevolen dagelijkse hoeveelheid foliumzuur voor een totale populatie. Ook indirect, via programmering van DNA door methylatie, mogen we verwachten dat foliumzuur groei en ontwikkeling beïnvloedt. Speculerend zouden eventuele schadelijke effecten door overstimulatie van de beschreven pathways door overmatige foliumzuurinname ook kunnen leiden tot ongeremde groei en versnelde ontwikkeling en mogelijk zelfs tot ontwikkeling van kanker.
Met de huidige resultaten kunnen nieuwe studies gestart worden teneinde de interactie tussen foliumzuur, de beschreven genen en eiwitten en het risico op CL/P verder te onderzoeken met als uiteindelijk doel het optimaliseren van preventie van CL/P en het beperken van de enorme impact bij aangedane families.

PhD Portfolio

Name PhD candidate: Bart J.B. Bliek
ErasmusMC Department: Obstetrics and Gynaecology
Promotors: Prof.dr. R.P.M. Steegers-Theunissen, Prof.dr. E.A.P. Steegers

Courses

- Course biomedical research techniques, ErasmusMC, Rotterdam	2004
- Master in Molecular Medicine, ErasmusMC, Rotterdam	2004, 2005
- BOP courses Pathology	2009, 2010
- Interstitial lung disease course, Davos	2011

Conferences

- Molecular Medicine day	2005, 2006
- Society for Gynecologic Investication – Annual Meeting, Los Angelos	2005
- 2nd Annual CMSB Members Symposium	2005
- Society for Gynecologic Investication – Annual Meeting, Toronto	2006
- RGOC onderzoeksdag / Wladimiroff symposium, ErasmusMC, Rotterdam	2006
- Pathologendagen, Zeist	2008, 2009, 2010

Presentations

- 2nd Annual CMSB Members Symposium, VU Medical Centre, Amsterdam (oral)	2005
- RGOC onderzoeksdag / Wladimiroff symposium, ErasmusMC, Rotterdam (oral)	2006
- Society for Gynecologic Investication – Annual Meeting, Los Angelos (1 poster)	2005
- Society for Gynecologic Investication – Annual Meeting, Toronto (3 posters)	2006

Teaching activities

- Pathology VO, ErasmusMC Rotterdam	2009, 2010

Curriculum Vitae

Bart Bliek was born in Rotterdam on November 7th, 1981. He passed secondary school at Comenius College in Capelle aan den IJssel where he graduated in 2000. In this year he attended Medical School at the Erasmus University Rotterdam and graduated in 2004 for his 'doctoraal' exam. From 2004 to 2006 he interrupted Medical School to work as a researcher at the Department of Obstetrics and Gynaecology, Division of Obstetrics and Prenatal Medicine of ErasmusMC in Rotterdam where the studies described in this thesis were performed. From September 2004 he attended the Master in Molecular Medicine and graduated in September 2005. From October 2006 to September 2008 he finished Medical School and obtained his MD degree. In April 2009 he started his residency in Pathology at the Department of Pathology of ErasmusMC, Rotterdam.
Bart is married to Anneke Monsma and father of Marieke.

Bart Bliek werd geboren in Rotterdam op 7 november 1981. Hij volgde zijn middelbare school op het Comenius College te Capelle aan den IJssel, waar hij slaagde in 2000. In datzelfde jaar startte hij met de studie Geneeskunde aan de Erasmus Universiteit Rotterdam en hij behaalde zijn doctoraal examen in 2004. Van 2004 tot 2006 onderbrak hij deze studie om als onderzoeker te werken op de afdeling Obstetrie en Gynaecologie, Divisie Obstetrie en Prenatale Geneeskunde van het ErasmusMC te Rotterdam, waar de studies beschreven in dit proefschrift werden uitgevoerd. Vanaf september 2004 volgde hij tevens de Master in Molecular Medicine waarvoor hij in september 2005 slaagde. Van oktober 2006 tot september 2008 liep hij co-schappen en voltooide daarmee de opleiding Geneeskunde. Vanaf april 2009 werkt hij als patholoog in opleiding op de afdeling Pathologie van het ErasmusMC te Rotterdam.
Bart is getrouwd met Anneke Monsma en vader van Marieke.

List of Publications

Bart JB Bliek, Annelies de Klein, Theo M Luider, Jan Lindemans, Lorette Hulsman, Coşkun Güzel, Christianne JM de Groot, Régine PM Steegers-Theunissen. New approach for the identification of folate related pathways in human embryogenesis. Cellular and Molecular Biology (2004), *50*, 939-944

Bart JB Bliek, Sheldon P Rothenberg, Régine PM Steegers-Theunissen. Maternal folate receptor autoantibodies and cleft lip and/or palate. International Journal of Gynecology and Obstetrics (2006), *93*, 142—143

Anneke Verkleij-Hagoort, **Bart JB Bliek,** Fakhredin Sayed-Tabatabaei, Nicolette Ursem, Eric AP Steegers, Régine PM Steegers-Theunissen. Hyperhomocysteinemia and MTHFR polymorphisms in association with orofacial clefts and congenital heart defects. A meta-analysis. American Journal Medical Genetics part A (2007), 143, 952-960

Bart JB Bliek, Régine PM Steegers-Theunissen, Leen Blok, Lindy Santegoets, Jan Lindemans, Ben Oostra, Eric AP Steegers, Annelies de Klein. Genome-wide pathway analysis of folate-responsive genes to unravel the pathogenesis of orofacial clefting in man. Birth Defects Research (Part A) 2008, *82*, 627–635

Bart JB Bliek, Ron HN van Schaik, Ilse P van der Heiden, Fakhredin A Sayed Tabatabaei, Cock M van Duijn, Eric AP Steegers, Régine PM Steegers-Theunissen and the Eurocran Gene-Environment Interaction Group. The risk of having a child with a cleft lip with or without palate and the maternal ABCB1 C3435T polymorphism and medication use. Journal of Medical Genetics part A (2009), *149A***,** 2088-2092

Bart JB Bliek, Coşkun Güzel, Annelies de Klein, Christoph Stingl, Theo M Luider, Jan Lindemans, Eric AP Steegers, Régine PM Steegers-Theunissen. Peptide fingerprinting of folate responsive proteins in human B-lymphoblasts and orofacial clefting. Submitted for publication

Bart JB Bliek, Régine PM Steegers-Theunissen, Hannie Douben, Jan Lindemans, Eric AP Steegers, Annelies de Klein. In vitro folate deficiency induces aneusomy of chromosome 17 and 21 without a relation to orofacial clefting in man. Submitted for publication.

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