# The search for risk factors that contribute to the etiology of non-syndromic cleft lip with or without cleft palate (CL/P) in the Polish population

Poszukiwanie czynników ryzyka, które mogą wpływać na powstawanie niezespołowych rozszczepów wargi połączonych, lub też nie, z rozszczepem podniebienia (CL/P) w populacji polskiej. Przegląd cyklu publikacji

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## STRESZCZENIE

Niezespołowe rozszczepy wargi połączone, lub też nie, z rozszczepem podniebienia (CL/P) zaburzają rozwój fizyczny i psychologiczny. W ostatnich latach dokonał się zauważalny postęp w zakresie wielospecjalistycznego leczenia CL/P, jednakże dalecy jesteśmy od zadowalającego wyjaśnienia etiologii rozszczepów. Nieprawidłowa palatogeneza jest powiązana z wieloma genami, szlakami metabolicznymi, niedoborami żywieniowymi oraz ekspozycjami na toksyny środowiskowe. Występują międzypopulacyjne różnice w udziale czynników ryzyka CL/P. Opracowanie zawiera przegląd prac oryginalnych autora i współpracujących z nim badaczy, które istotnie przyczyniły się do poznania zarówno środowiskowych, jak i genetycznych czynników ryzyka CL/P w populacji polskiej. Wyniki badań sugerują zależność ryzyka CL/P od matczynych zasobów α-tokoferolu, retinolu, cynku, cytruliny oraz składników diety wpływających na homeostazę grup metylowych. Polimorficzne warianty genów *BHMT1* i *BHMT2* są istotnie statystycznie powiązane ze zmniejszonym ryzykiem CL/P w populacji polskiej. Wykazano również zależności pomiędzy genami *MTR, PCYT1A, ASS1, SLC25A13, GSTM1, GSTT1, SUMO1* oraz loci 8q24.21 a nieprawidłową palatogenezą. Objęte przeglądem prace oryginalne umożliwiły identyfikację szlaków metabolicznych i nowych genów kandydackich, które stanowią interesującą podstawę do dalszych badań, oraz potwierdziły niektóre wcześniejsze obserwacje dotyczące czynników usposabiających do rozszczepów twarzoczaszki.


**Słowa kluczowe:** rozszczep wargi, rozszczep podniebienia, czynnik ryzyka, żywienie, geny kandydackie


## ABSTRACT

Non-syndromic cleft lip with or without cleft palate (CL/P) has severe long-lasting adverse effects on both physical and psychological development. Although recent years have brought significant improvement in clinical treatment, our understanding of the etiology of this congenital anomaly is lagging. Many genes, genetic pathways, harmful exposures, as well as nutrient deficiencies have been implicated in abnormal palatogenesis. Extrapolating data regarding risk factors for CL/P from different populations is not always straightforward. This review gives an overview of knowledge about both environmental and genetic risk factors in the etiology of CL/P in the Polish population, which was originally presented in research papers by the author and collaborating investigators. The findings suggest a possible association between maternal consumption of α-tocopherol, retinol, zinc, citrulline, and dietary compounds contributing to methyl-group homeostasis and CL/P risk. Polymorphisms in *BHMT1* and *BHMT2* were significantly associated with decreased CL/P risk in the Polish population. Other interesting findings include associations of CL/P risk with polymorphisms in *MTR, PCYT1A, ASS1, SLC25A13, GSTM1, GSTT1, SUMO1* genes, and 8q24.21 loci. Reviewed research papers indicate interesting pathways and new candidate genes for further investigation, and partly confirm earlier findings according risk factors for orofacial clefts.


**Key words:** cleft palate, cleft lip, risk factor, nutrition, candidate genes

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Clinically, cleft lip is an unilateral or bilateral gap between the philtrum and the lateral upper lip, often extending through the upper lip and jaw into the nostril. Approximately 70% of cleft lips are accompanied by a cleft of the secondary palate, which constitutes the roof of the oral cavity [1, 2]. The presence of an orofacial cleft has severe and long-lasting adverse effects on both physical and psychological development and imposes a substantial social and economic burden. In the United States, for example, the lifetime cost for treating orofacial clefting has been estimated to be approximately $US101,000 [3]. Prevention of abnormal palatogenesis has been hampered by a shortage of information about modifiable risk factors. Non-syndromic cleft lip with or without cleft palate (CL/P) is one of the most common human birth defects, with an average worldwide prevalence of 1.2/1,000 live births [1, 2, 4]. In Poland, the rate of occurrence of this common malformation is 1.7/1,000 [5]. The incidence correlates with geographic origin, racial and

ethnic background. Concordance of orofacial clefts in monozygotic twins ranges between 40% and 60%, suggesting a role for environmental factors and exposure conditions i.e. nutritional deficiencies, toxins, physical constraint *in utero*. Increased phenotypic variances and asymmetry for craniofacial measurements in parents of CL/P-affected children, as well as high recurrence risks (20–30 times greater than population prevalences) provide evidence for a strong genetic component to clefting. Since the mother is the environment of the developing embryo, interactions between genetic and lifestyle factors are assumed to be involved in abnormal palatogenesis. Based on experimental and epidemiological data, CL/P etiology is considered to be complex, multifactorial, and determined by numerous interacting gene loci with additional environmental covariates [1, 2, 6–11].

In the human genome, only a difference of about 1.6% between modern humans and the most developed primates has been found. In contrast, human dietary habits have markedly evolved since origin, about 2–7 million years ago, especially during the last century. The per capita consumption of refined sugar has increased from 0.5 kg/year in 1850 to about 50 kg/year in the recent decade. The concept of environment is complex and broad, and it has been frequently associated with pollutants, infections, risky behaviors, etc. However, food intake is the environmental factor to which we are all permanently exposed from conception, and it has been a major driving force through species' evolution [12]. Therefore, dietary habits and nutrient intakes are the most important environmental factors modulating gene expression during one's life span. The several lines of evidence support an association between maternal nutrition and risk of clefting in offspring [4, 13]. However, in the majority of individuals with CL/P a specific causative agent cannot be identified, and the detailed proportion of cases of clefts that are potentially preventable through changes in maternal nutrition and other lifestyle choices is currently unknown. The contribution of different etiologic factors may vary from patient to patient. On one end of the spectrum we can find genetic factors leading to an orofacial cleft without any significant environmental involvement. In other cases, genetic factors may provide a background that makes an individual susceptible to the development of the anomaly. For other patients, environmental factors may play a large role in the etiology of orofacial cleft [8–11].

Because past research indicates that most cases of *spina bifida* are preventable, identifying the contribution by which modifiable risk factors in the environment influence the risk of other structural malformations is important [11, 14]. There is an agreement in the literature regarding the need for identification of the specific factors which predispose an individual to abnormal palatogenesis as an important step leading to a reduction of the disability [9, 11, 15]. The relationship between maternal dietary intake and embryonic/fetal nutrition is not fully understood. Nutrient supply to the embryo can be influenced by a number of adaptive physiological changes that occur during pregnancy, including alternations in maternal intestinal absorption, and transfer mechanisms. Environmental exposures act through their impact on the mother and embryo and they can be studied using markers of exposure but also of susceptibility [4]. Variations in single nucleotide polymorphisms (SNPs) can have functional consequences ranging from severe to none. Variants can either increase or decrease case risk. In most individuals, these variants do not adversely affect the phenotypic appearance of their carrier. In others, however, a single gene variant or a combination of SNPs may lead to effects that exceed our normal structural variations. The risk of CL/P is expected to be heavily influenced by the patterns of SNPs [7–9]. Among various common types of alternation in DNA sequence such as insertions (e.g. cystathionine-beta synthase CBS 844ins68), deletions, and large-scale copy-number variations, SNPs are the most usually studied. The technology for detecting many SNPs in large populations has become feasible and affordable [4, 12].

However to date, there are no published reviews of studies devoted to genetic polymorphic variants as well as nutritional risk factors contributing to the etiology of orofacial clefts in the Polish population. Unfortunately, extrapolating data according to risk factors for CL/P from different populations is not always straightforward. Differences in risk estimates for candidate genes and environmental risk factors can be caused by etiologic heterogeneity between populations, differences in ethnic background and lifestyle [15–17]. Variation of CL/P expression in ethnic groups indicates genetic differences in susceptibility. Therefore, this review summarizes the most important observations on inherited, nutritional, and metabolic CL/P risk factors in the Polish population, which were presented by the author and collaborating investigators [18–33].

## I. Environmental factors

The role of nutrition in the etiology of non-syndromic orofacial clefts has been appreciated since the beginning of 20$^{th}$ century, when *Strauss* suggested a possible link between diet without fresh meat for jaguars, and delivering cubs with a cleft palate [34]. We are living in a society that is over-fed and undernourished, with deficiencies apparent from a so-called "well-balanced" diet. This topic is the subject of an excellent recent review by *Glenville* [35].

**Lipid-soluble vitamins**

Vitamin E deficiency-associated teratogenicity has been suggested by Cheng and Thomas in 1952 [36]. A significant reduction of the incidence of maternal diabetes-related fetal malformations including orofacial clefts has been reported in rodents supplemented with vitamin E [37]. In a study aimed to evaluate the association between vitamin E and clefting, the ratio of α-tocopherol to total serum cholesterol were analyzed in 26 mothers of children with isolated cleft lip and 36 control mothers [20]. The ratio, as well as α-tocopherol level in erythrocytes, was significantly lower in Polish mothers of cleft-affected children. Interestingly further studies on vitamin E in mothers of children with CL/P showed: 1) The distribution of results to the clusters was significantly dependent on type of the cleft: isolated cleft lip or cleft lip with cleft palate (p=0.03), which may suggest etiological distinction between them [38]; 2) The multiple linear regression model with body mass index (BMI), $BMI^2$, age, concentration of plasma retinol, and fish consumption as independent variabs predicted a 40% of variance in the plasma α-tocopherol concentration [39]. These findings indicating a variance of α-tocopherol concentration should be considered in future studies. It has not yet been proven whether the teratogenic effects of an α-tocopherol deficiency are due directly to a deficiency of the vitamin, or whether they indirectly occur through modulators associated with α-tocopherol homeostasis. Moreover, future studies are recommended to test whether isolated cleft lip and cleft lip and palate have distinct etiologies, which has also been suggested by other investigators [40].

Maternal intake of vitamin A from supplements >10,000 IU has been shown to cause CL/P in addition to other malformations [15, 41]. Vitamin A intoxication results in a multitude of alternations in mammalian embryos and several genes involved in palate development (i.e. muscle segment homeobox homolog 1, MSX1 and transforming growth factor β3, TGFβ3) interact or can be modified in expression by vitamin A and its analogs [41]. It is noteworthy that among unsupplemented Polish women high plasma retinol levels, exceeding the upper laboratory norm, were detected in mothers of children with orofacial cleft at two times that of control mothers, 11.5% (11/96) vs. 5.8% (3/52), respectively [19]. The decision regarding periconceptional vitamin A supplementation should take into account the retinol status of the women [19, 41, 42].

**Water-soluble vitamins**

One of the most important discoveries in reproductive medicine is the possibility that periconceptional intake of supplements with water-soluble vitamins may reduce the risk of CL/P in offspring, similar to the known risk reduction for spina bifida seen with folic acid [11]. However, it must be noted that findings from case-control studies into the use of multivitamin supplements (Fig.1), dietary folate intake, and folate levels in blood are inconsistent [14]. Polish mothers who gave birth to babies with CL/P tended to use less vitamin supplements during pregnancy than control mothers [18]. In the years 2001-2002 only approximately 3% of mothers declared the use of folate supplements during the preconceptional period [18]. Thus, efforts to increase awareness of a healthy diet and lifestyle should be strengthened not only throughout pregnancy but also before, given that in Poland pregnancies are often unplanned [43].

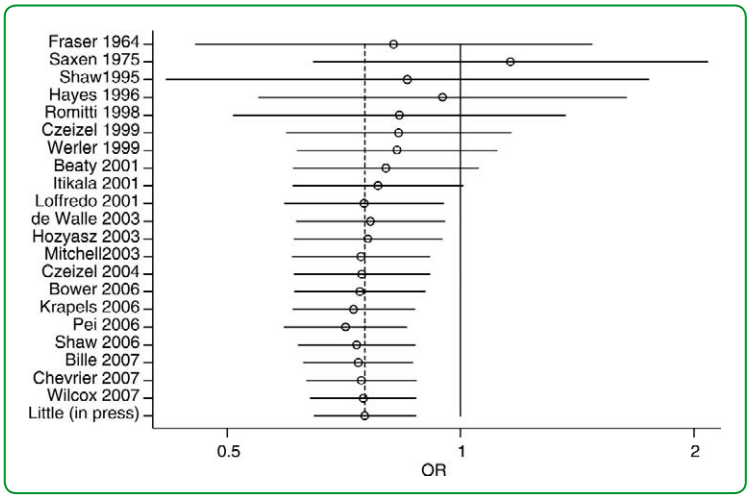


**Fig. 1. Random effects cumulative meta-analysis showing the association between supplement use before or during pregnancy and the risk of CL/P over time as OR and 95% CI (Adapted from Johnson and Little [14], with permission from Oxford University Press)**

**Ryc. 1.** Metaanaliza wyników badań nad powiązaniem stosowania suplementów witaminowych przed i w czasie ciąży a ryzykiem CL/P u potomstwa. Zobrazowano względne ryzyko (OR) i 95% przedział ufności (CI). Adaptowano z Johnson i Little'a [14], z pozwoleniem Oxford University Press

The underlying process by which folic acid may alter the risk of abnormal palatogenesis in humans is unknown, one suggested mechanism for folate's preventive role involves methyl group donors [9,11]. Imbalances of folate methyl donor and vitamin B12 (cobalamin) cofactor play a crucial role in disturbing the one-carbon metabolism [11]. A low maternal vitamin $B_{12}$ status was reported to be associated with a higher risk of CL/P in the Dutch [44]. There are two reactions that require derivatives of vitamin $B_{12}$ for activity: the cytoplasmic enzyme methionine synthase (MTR) and the mitochondrial enzyme methylmalonyl-CoA mutase. Decreased activity of methylmalonyl-CoA mutase results in the accumulation of methylmalonyl-CoA and propionyl-CoA. Excess of propionyl-CoA is converted to propionylcarnitine (C3). Therefore, high levels of propionylcarnitine may serve as a marker of vitamin B12 deficiency. The

study investigating propionylcarnitine levels in Polish newborns with CL/P showed that a deficiency of vitamin B12 with metabolic disturbances seems not to be a risk factor for orafacial clefts in an enrolled group of 52 patients [29]. The mean concentrations of whole blood propionylcarnitine in newborns with CL/P and controls were 2.82 µmol/L (SD 1.06) and 2.68 µmol/L (SD 0.94), respectively (p>0.05).

Maternal biotin (vitamin H) deficiency is teratogenic in rodents. Moreover, this deficiency is one of the most potent clefting factors even when the dams do not show any signs of biotin deficiency. Similar pathologic signs and symptoms of advanced biotin deficiency such as alopecia, dermatitis, and neurologic abnormalities develop in both rodents and humans. Zempleni and Mock [45] suspected that the following factors might predispose humans to fetal malformations caused by biotin deficiency: 1) Frequent spontaneous maternal vitamin H deficiency of a marginal degree; 2) Weak placental biotin transfer; 3) An increased biotin requirement of proliferating cells. However, the strict mechanisms by which biotin deficiency produces specific abnormalities are lacking. Both acetyl-CoA carboxylase 1 (ACC1) and acetyl-CoA carboxylase 2 (ACC2), which are crucial biotin-dependent enzymes, catalyze the incorporation of bicarbonate into acetyl-CoA to form malonyl-CoA. The malonylcarnitine level might reflect malonyl-CoA homeostasis. In Polish newborns with CL/P low malonylcarnitine levels (≤ 0.047µmol/L) were 1.7 times more predominant than in healthy individuals, p=0.03. The findings may suggest that the metabolic pathway of malonyl-CoA is disturbed in CL/P-affected individuals, however the potential role of biotin-dependent carboxylases has yet to be elucidated [28]. Moreover, further studies are needed to clarify the relation between maternal carnitine (so-called vitamin $B_T$, which is a hydrophilic molecule) and its derivatives (e.g. acylcarnitines) status and clefting risk [28, 46]. Carnitine plays an indispensable role in fatty acid oxidation. It is noteworthy that there is strong evidence for the utilization of lipids as an energy substrate by early embryos [47]. The formation of acylcarnitine conjugates is the basis of expanded newborn screening for inborn errors of metabolism based on tandem mass spectrometry (MS/MS).

**Minerals**

The functions of zinc in the human and experimental animals' reproduction have been studied extensively and reviewed recently by Shah and Sachdev [48]. At least in rodent models in the face of an acute dietary zinc deficiency, maternal mobilization of zinc stores is inadequate to supply the needs of the conceptus. In rats the deficiency of zinc results in offspring that are characterized by anomalies affecting nearly every organ. In the years 2004-2005 low zinc level was independently reported as a maternal risk factor for orofacial clefts in the Netherlands (in erythrocytes) [49], the Philippines (in plasma) [50], and Poland (in serum) [22]. In mothers of children with CL/P mean serum zinc level was lower than in women who gave birth to children without a birth defect, 511µg/L (SD 121) vs. 572 µg/L (SD 76), p=0.01, respectively [22]. The second Polish study, in which zinc was analyzed in whole blood, confirmed an association between low maternal zinc and increased risk of CL/P in offspring [25]. A maternal whole blood zinc concentration of 47.1µmol/L or less increased the risk of CL/P 2.5-times more than higher concentrations (95%CI:1.03-6.23, p=0.04).

Zinc transporters SLC30A1 and SLC30A5 play a key role in regulation the delivery of maternal zinc to the developing embryo. Embryonic nutrition is determined not only by the mother's dietary intakes and nutrient stores, but also by transfer capabilities. Cadmium exposure down-regulates *Slc30a1* expression, indicating that maternal cadmium exposure may alter zinc homeostasis in the conceptus [51]. Experimental and epidemiological studies have reported an association between prenatal exposure to cadmium and structural malformations [51, 52]. In contrast to the study by Cesany et al. conducted in Moravia and Silesia [53], we found no significant association between cadmium exposure and the risk for orofacial clefts in offspring [52]. There is increasing evidence for an interaction between zinc, cadmium, and iron during intestinal absorption [54]. Moreover, the secondary findings of the study by Czeizel et al. [55] showed a lower risk of cleft palate in pregnant women with iron supplementation. However, we failed to find an association between maternal serum iron and risk for CL/P [56]. Animal models have shown that copper intoxication in early pregnancy results in abnormal embryogenesis. It is noteworthy that a combination of low whole blood zinc and high copper concentrations was seen only in Polish mothers of children with CL/P, but not in control mothers (4/116 vs. 0/64, respectively) [25].

Naturally grown produce is a richer source of trace elements such as zinc than similar cultivated produce. Red meat is frequently regarded as an unhealthy food and it's low intake is often recommended. It is not taken into account that red meat is important for some micronutrients such as zinc and vitamin $B_{12}$. Zinc from animal sources is belived to be most bioavailable. Increased total preconceptional zinc intake was associated with a reduced risk for neural tube defects in California [57]. It is reasonable to consider zinc supplementation in women of childbearing age, because zinc can be administered easily and safely, is well tolerated and inexpensive. Additional studies,

however, are needed to identify whether zinc supplementation in the periconceptional period results in functional and measurable outcomes for offspring.

**Citrulline**

The non-essential amino acid citrulline is poorly represented in food except in Cucurbitaceae fruits and birch sap, which have both been used in the treatment of reproductive disorders for centuries. Retrospective analysis of citrulline concentrations obtained from the results of the Polish Newborn Screening Program for Inborn Errors of Metabolism based on MS/MS revealed that low whole blood citrulline levels were three times more predominant in newborns with CL/P than in healthy individuals, 5/52 (10%) vs. 3/107 (3%), respectively. On the other hand, high levels of citrulline were observed nearly two times more frequently in the control group than in patients with CL/P, 43/107 (40,2%) vs. 12/52 (23,1%), p=0.03 [26]. The integration of this study data with the existing literature suggests that maternal citrulline intake may contribute to reduced risk of abnormal embryogenesis [26]. The findings from the "citrulline" study provided important insights about citrulline/arginine-related genes as potential candidate genes for CL/P [26,30]. The findings have led to suggestions that an increased intake of citrulline may reduce birth defects risks. Modern humans have primate ancestors and probably differ little from them biologically. By mixing plant food from a variety of species each day, wild apes are able to upgrade their overall dietary quality in terms of complementary amino acids as well as vitamins and minerals [58]. Both the amount of food consumed and the composition of the diet are important.

**BMI and other life-style factors**

Potential environmental risk factors for CL/P include maternal characteristics that impact the *in utero* environment of the embryo. The achievement or maintance of an ideal body weight improves pregnancy outcomes. A number of studies have examined the association between maternal prepregnancy BMI and CL/P and other birth defect risks in West European and North American populations, although findings have been inconsistent [59]. Offspring of investigated Polish mothers with low prepregnancy BMI (<19.8kg/m$^2$) are at an increased risk for isolated cleft lip [60]. Women with low BMI might have a nutritional deficit, resulting from poor-quality diets or dieting behaviors. No increased risk was found for CL/P in relation to maternal obesity in Poland [60]. BMI, as well as smoking status, may influence vitamin status of mothers of CL/P-affected children [42, 60–63]. Differences have been seen between smokers and non-smokers for preconceptional and prenatal care utilization in Poland [62]. Increasing access to prenatal care is regarded as one of the key elements for promoting positive nutrition practices among women during pregnancy.

## II. Variants of reported and new candidate genes

Candidate genes for CL/P were chosen from several sources such as genes responsible for syndromic malformations (e.g. van der Woude syndrome - interferon regulatory factor 6, IRF6), genes that are linked to congenital malformations in animal studies (e.g. cleft palate in Tgf-β3 knockout mice), genes that are part of pertinent biological pathways (e.g. folate pathway genes, biotransformation of toxic compounds), and analyzes of gene expression in human and rodent embryonic tissues [4,64]. Analyzes of candidate loci and genome-wide linkage scans reported in the literature have shown a wide range of plausible genes or regions for orofacial clefts. However, genetic findings presented in the literature can explain only a small proportion of the genetic component contributing to the pathogenesis of CL/P [4,9].

**Folate pathway**

The main concept in nutritional genetics is that some minor alternations in gene sequence can modulate, to some extent, specific metabolic pathways which make the corresponding subjects more or less prone to respond to dietary intakes and influence the risk of abnormal embryogenesis. The intracellular concentrations of the different folates are in general much lower than their Michaelis constant values for the enzymes, and so the rate or steady state of the reaction can change over quite a large range of cellular folate concentrations. A number of investigators studying orofacial clefts have concentrated on the folate pathway because it is well known that periconceptional folic acid supplementation may reduce the risk for structural malformations. Methylenetetrahydrofolate reductase (MTHFR) catalyzes the reduction of 5,10-methylenetatrahydrofolate to 5-methyltetrahydrofolate, the predominant circulatory form of folate and methyl donor for the remethylation of homocysteine to methionine. The *MTHFR* rs1801133 (c.677C>T) is the most intensively investigated variant in the homocysteine/folate pathway [11, 14, 65]. However, results of the *MTHFR* rs1801133 association in different CL/P populations are inconsistent (Fig. 2), indicating the challenges of researching gene-disease associations [14]. Both fetal and maternal genetic susceptibilities may affect the intrauterine environment during palatogenesis. We found no association between maternal, as well as embryonic, *MTHFR* rs1801133, and *MTHFD1* (gene encoding trifunctional enzyme methylenetetrahydrofolate dehydrogenase 1) rs2236225 (c.1958G>A) and CL/P risk [24,32]. Maternal RFC1 (reduced folate

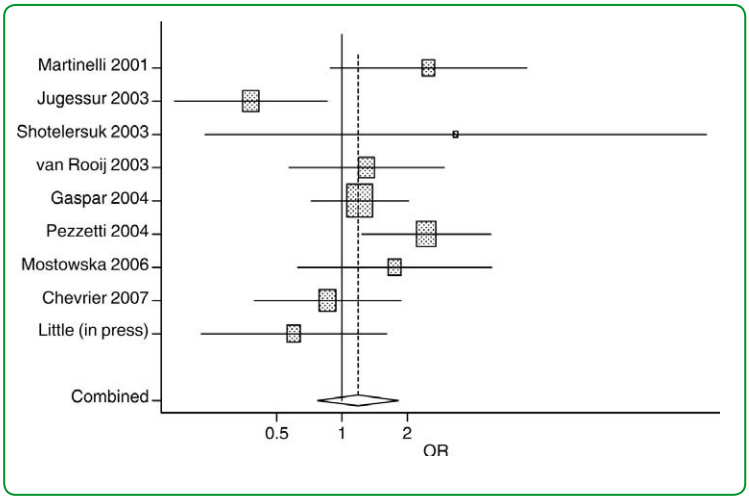


**Fig. 2. Random effects meta-analysis of the association between maternal MTHFR C677T TT versus CC genotype and CL/P showing OR and 95% CI (Adapted from Johnson and Little [14], with permission from Oxford University Press)**
**Ryc. 2.** Metaanaliza powiązań genotypu matki MTHFR C677T TT w porównaniu do CC z ryzykiem CL/P u potomstwa. Zobrazowano względne ryzyko (OR) i 95% przedział ufności (CI). Adaptowano z Johnson i Little'a [14], z pozwoleniem Oxford University Press

carrier 1) rs1051266 (c80A>G) and embryonic *MTR* rs1805087 (c.2756A>G), *MTRR* (methionine synthase reductase) rs1801394, CBS 844ins68, *TCN2* (transporter transcobalamin II) rs1801198 were not correlated with CL/P susceptibility in the Polish population [23, 31].

Genetic processes that alter gene function without structural DNA alternation have become one of the chief focus areas of developmental medicine. Recently, there has been increased interest in epistasis and its influence on congenital anomalies in general. The nonparametric and genetic model-free Multifactor Dimensionality Reduction (MDR) analysis revealed a significant interactive effect of investigated SNPs in embryonic genes encoding enzymes involved in one carbon metabolism on clefting susceptibility (i.e. *MTHFR* rs1801133, *MTR* rs1805087, and *PEMT* /phosphatidylethanolamine N-methyltransferase/ rs4646406 – a testing balance accuracy of 0.62 and a cross-validation consistency of 6/10, p=0.02) [31]. Even in the absence of an independent effect on CL/P risk in the Polish population, the presence of the *MTHFR* rs1801133 may result in an increased CL/P risk. Studies using a variety of approaches have produced conflicting or inconclusive results on the MTHFR rs1801133 in clefting susceptibility, possibly because of the diversity of the investigated populations or the inadequate power of the studies.

It is especially noteworthy that Polish mothers homozygous (GG) or heterozygous (AG) for the top-SNP of *MTR*, rs1805087, displayed a twofold increased risk of having a child with CL/P ($OR_{AG+GGvsAA}$=2.19, 95%CI=1.19-4.05, p=0.01) [23]. Interestingly, maternal genotypes that include the G allele have also been associated with an increased risk of neural tube defects and conotruncal heart defects [66, 67]. Methionine synthase, encoded by *MTR*, is a vitamin $B_{12}$-dependent enzyme that functions within the transmethylation cycle by catalyzing the 5-methyltetrahydrofolate-dependent remethylation of homocysteine to methionine.

**Genes for transcription factors, growth factors, receptors, and other reported risk factors**

The purpose of the study published in the Birth Defects Research [27] was to investigate the contribution of 18 polymorphic variants of the 10 most prominent, previously reported, candidate genes to abnormal palatogenesis in a sample of Polish patients with CL/P. A statistically increased risk of CL/P was observed for SNPs located in the 8q24.21 region (rs987525 $OR_{AC+AAvsCC}$=1,96; 95%CI=1.38-2.78, p after correction for multiple testing /$p_{corr}$/=0.002), IRF6 (rs642961 $OR_{AG+AAvsGG}$=1.63, 95%CI=1.1.15-2.31, p=0.005) and SUMO1 (small ubiquitin-like modifier 1; rs2350358$OR_{CGvsGG}$=1.58, 95%CI=1.06-2.36, p=0.03) locus, but not for genes encoding transcription factors like MSX1, PAX9 (paired box 9), TBX10 (T-box transcription factor 10), FOXE1 (forkhead box E1); growth factors TGFα (transforming growth factor α), TGFβ3, FGF10 (fibroblast growth factor 10), and receptor FGFR1 (fibroblast growth factor receptor 1). Recent studies based on genome-wide association analyses have reported a key susceptibility locus for CL/P on chromosome 8q24.21. Interestingly the 8q24.21 region does not contain any known genes. The study on Polish patients with CL/P replicated the previously reported association between the 8q24.21 rs987525 and clefting in the neighboring populations of Germany, Estonia, and Lithuania, as well as Irish, non-Hispanic whites from the US, Mayan Mesoamerican population, and Asians [16, 68–71]. The frequently studied candidate gene that has been found to be strongly associated with CL/P is IRF6. This association has been confirmed in multiple populations. However, *IRF6* does not account for the majority of the genetic contribution to CL/P [72]. SUMO is a small protein that can be covalently linked to specific proteins, including the products of developmental genes with evidence of having a role in abnormal palatogenesis (e.g. *MSX1, PAX9*), as a posttranslational modification. On the other side, the process of sumoylation 1 is also known to be susceptible to environmental effects linked to increased risk of CL/P, e.g. oxidative stress. DNA is a major target of constant oxidative damage from endogenous oxidants. Levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in DNA are a balance between formation and repair of this oxidative damage. 8-OHdG is continuously excreted into the

bloodstream. Interestingly, 1 to 6 months after delivery of children with orofacial clefts, increased serum concentrations of 8-OHdG were reported in Polish mothers [73, 74].

**Polymorphisms located in the region containing *BHMT1* and *BHMT2* genes**

One goal of nutritional genomics is to find markers that reveal significant gene-diet interaction, thus providing tools for personalized and more successful dietary recommendations ("nutrigenomics") [12]. Betaine was first discovered by a German chemist Scheibler in the juice of sugar beets in the 19$^{th}$ century. Mammals use betaine for three key functions: 1) A methyl donor for the remethylation of homocysteine to methionine; 2) The major organic osmolyte; 3) A regulator of lipid metabolism. Choline is committed to become a methyl donor after it is oxidized to form betaine in the inner mitochondrial membrane. The alternative choline/betaine-dependent pathway for the methylation of homocysteine to form methionine is catalysed by betaine homocysteine methyltransferase 1 (the product of the *BHMT1* gene, also known as *BHMT*), which is a zinc metalloprotein. Humans obtain betaine from foods that contain either betaine or choline-containing compounds. It is probable that most of the body's betaine needs can be met by choline oxidation. On the other side the body can produce de novo choline via *PEMT*, however it costs three methyl groups to do so and this pathway seems not to represent a net increase in available methyl groups. The existence of multiple mechanisms, which ensure the availability of choline to the fetus (i.e. the placenta stores large amounts of choline as acetylcholine), suggest that evolutionary pressures favored exposure to high concentrations of choline in utero. Since choline oxidation to betaine is irreversible it diminishes the availability of choline for its other vital functions, and therefore dietary betaine spares choline and may be essential during pregnancy to ensure adequate choline for phospholipid and neurotransmitter synthesis [75]. Since epidemiological studies have provided us with data reflecting the harmful effects of maternal alcohol use on palatogenesis [15, 76], it is worth noting that alcohol is reported to inhibit MTR, increasing the requirement for betaine to sustain methylation [77]. Embryonic alcohol effects are preventable by abstinence during pregnancy but often unavoidable because many pregnancies are unplanned and hence alcohol consumption occurs before a woman knows that she is pregnant [43]. In experimental studies betaine has been clearly shown to have an important role in early mammal development [78]. The best dietary sources of betaine are beets (*Beta vulgaris* has three basic varieties; chard-spinach beet, beets-red, yellow or white, and sugar beets), spinach, wheat bran and germ, shrimps and other seafood. Examples of food with high choline content are eggs, liver, red meat, and wheat germ. *Zeisel* [79] suggested that significant variation in the dietary requiment for choline can be explained by very common genetic polymorphisms. Analysis of two SNPs in the *BHMT1* gene, rs3733890 and rs585800, revealed that these SNPs' allele and genotype frequencies have significant differences between CL/P–affected individuals and controls (p=0.012, p=0.002 and p=0.011, p=0.024, respectively). Individuals with the rs3733890 AA genotype have a significantly lower risk of CL/P ($OR_{AAvsGG}$=0.14; 95%CI:0.04-0.48, p=0.0004, $p_{corr}$=0.0054) [31]. The *BHMT1* polymorphisms rs3733890 and rs585800 are significantly correlated with each other in the Polish population. Interestingly, none of the investigated five SNPs of maternal BHMT1, including rs3733890, rs585800 and rs3733890, were associated with case-control status after correction for multiple testing [32]. Recently, Hobbs et al. [80] reported maternal obesity in combination with the *BHMT1* rs3733890 may increase the risk of congenital heart defects. Betaine has been used as a dietary supplement in animal husbandry for 60 years, because it protects from the osmotic stress, maldigestion, as well as increases the lean muscle mass in pigs and ruminants.

S-methylmethionine, also known as vitamin U and anti-ulcer factor, was first isolated by McRorie et al. in 1953 [81]. In plants vitamin U plays a role as a reserve form of methionine and osmoprotectant. The best dietary sources of SMM are *Brassica vegetables* (i.e. Western cabbage, China Cabbage, and broccoli), garlic, soy bean, sweet corn, and celery. In animal models SMM has choline- and methionine-sparing activity. *BHMT2* is a zinc metalloenzyme that methylates homocysteine using SMM. Unfortunately, very little data are available from functional genomic studies on *BHMT2*. Recently, *Bhmt2* was identified as a diet-dependent genetic factor protecting against acetaminophen-induced liver toxicity [82]. The BHMT2 rs625879 TT homozygous mothers had a decreased risk of having CL/P offspring compared to women with the GG genotype ($OR_{TTvsGG}$=0.31; 95%CI: 0.16-0.63, p=0.0009 and $p_{corr}$=0.02). In mothers, but not in affected patients, we observed weak influence of the *BHMT2* rs526264 on CL/P risk [31, 32]. *BHMT2* rs625879 and rs526264 are strongly correlated. The mechanisms by which polymorphisms of the BHMT1 and *BHMT2* genes might influence the susceptibility to CL/P requires further investigation. The high linkage disequilibrium across the chromosomal region containing *BHMT1/2* makes it difficult to distinguish a real genetic risk factor. Moreover, it is possible that all associations of the CL/P candidate genes observed in reviewed papers represents indirect associations with other polymorphisms, genes or regulatory elements.

Outlets selling supplements for humans offer betaine usually labeled as trimethylglycine or TMG. SMM is marketed as herbal medicine in Asia and Stomacin U® tablets in the United States.

**Other polymorphic variants of genes involved in choline metabolism**

The choline and folate metabolic pathways are interrelated and intersect at the step of methionine formation from homocysteine [83]. We analyzed polymorphisms of *PEMT* and genes encoding choline kinase (CHKA), choline dehydrogenase (CHDH), and choline-phosphate cytidiltransferase A (PCYT1A). For the investigated 5 polymorphisms of *CHKA*, *PEMT*, and *CHDH* in CL/P-patients there was no evidence for both allelic and genotyping association with the risk of a being a case; however, other variants in these genes should be examined for a possible role in oral clefts [31]. It is noteworthy that embryonic *PCYT1A* rs7639752 A allele increased the risk of having CL/P-affected offspring nearly twofold in the Polish population ($OR_{AG+AAvsGG}$=1.89; 95%CI: 1.15-3.11; p=0.01); however, the results were not statistically significant after adjustment for multiple comparisons [31]. The PCYT1A protein is a rate controlling enzyme in the choline pathway [84]. The gene-gene interaction analysis revealed a significant epistatic interaction of the *BHMT2* rs673752, *PCYT1A* rs712012, and *PEMT* rs12325817 with maternal CL/P susceptibility (p=0.019) [32]. Thus, this may suggest that common SNPs in genes of choline metabolism may influence the demands for SMM as a methyl-group donor. Proper interpretation of the presented results on gene-gene interaction await further studies.

**Genes related to arginine/citrulline metabolism**

There is a growing body of evidence that homeostasis of amino acids from the arginine family may play an important role in early human development [85]. Aberrant metabolism in environmentally sensitive pathways in individuals with CL/P who have no known metabolic disease is of growing interest [26]. A moderate association between polymorphic variants of genes for enzymes constituting an arginine-citrulline cycle and risk of clefting was demonstrated in the study of Polish CL/P-affected patients [30]. The calculated OR for individuals with the gene for argininosuccinate synthetase 1 (*ASS1*) polymorphism rs7860909 G allele compared to AA homozygotes was 1.768 (98%CI: 1.133-2.759; p=0.01). MDR analysis provided evidence of interaction between the genes ASS1, a liver-type mitochondrial aspartate-glutamate carrier (*SLC25A13*), and argininosuccinate lyase (ASL) on CL/P susceptibility [30]. The overall best MDR model included two polymorphisms (the ASS1 rs 666174 and SLC25A13 rs10252573). This model had a testing balance accuracy of 0.64 and a cross-validation consistency of 9/10 (p=0.002). Deficiency of citrin, a liver-type mitochondrial aspartate-glutamate carrier leads to a quantitative deficiency of *ASS1* without any detectable abnormalities in the *ASS1* gene or ASS1 mRNA levels. We believe this is the first study to evaluate DNA sequence variants in the human *ASS1*, *ASL* and *SLC25A13* genes for a possible association with a structural malformation risk. These novel findings suggest a crucial role for arginine/citrulline-dependent metabolic pathways in the early human development, table I. Moreover, it is important for future investigations to consider entire gene families and those in which they interact.

**Glutathione transferase gene family**

There are several complex enzymatic mechanisms to detoxify a wide array of xenobiotics absorbed by ingestion, inhalation, or surface contact. Maternal smoking is an established risk factor for CL/P [34,61]. S-glutathione transferases affect the detoxification of different compounds including those from cigarette smoke. Our group recently examined genes for S-glutathione transferase M1 (*GSTM1*) and S-glutathione transferase T1 (*GSTT1*), which conjugate glutathione with xenobiotics and promote their removal from the human body [21]. The frequency of the homozygous *GSTM1* and *GSTT1* deletions varies across populations. A significantly increased risk of giving birth to a child with CL/P was found in multiparous mothers with *GSTM1(-)/GSTT1(-)* and *GSTM1(-)/GSTT(+)* genotypes as compared to those with *GSTM1(+)/GSTT1(+)* genotype (OR=6.96; 95%CI:1.15-8.08, p<0.02), however, no gene-smoking interaction effects were identified. Thus, our study showed gene-only effects but did not observe gene-environment interaction. The role of polymorphic variants of genes for S-glutathione transferases in abnormal palatogenesis was reported in several studies from Western Europe and the United States [86].

**Genes encoding enzymatic antioxidants and zinc transporters**

Antioxidants are present in both enzymatic (i.e. catalase (CAT), glutathione peroxidase (GPX), and superoxide dismutase (SOD)) and non-enzymatic forms (i.e. vitamin E, zinc) forms. Zinc is involved in the antioxidant defense as a cofactor of enzymes (i.e. in metallothionein and Cu,ZnSOD) and counteract oxidation through binding sulphydryl groups in proteins and by occupying binding sites for iron and copper in lipids, proteins and DNA. Reactive oxygen species are produced under physiological and pathological conditions and are involved in signal transduction and gene transcription. They are suggested

**Table I. Nutrition and related genetic factors, which form promissing areas for further research on environmental-gene interactions in the Polish population and future CL/P prevention triais based on increased intake of selected nutrients**
**Tabela I**. Czynniki żywieniowe i genetyczne, które stanowią obiecujący obszar badań nad powiązaniami środowiskowo-genetycznymi w populacji polskiej oraz zapobieganie CL/P na drodze wzbogacania diety

| Nutrient<br>Składnik diety | Dietary source<br>Najzasobniejsze pokarmy | Metabolic pathway<br>Szlak metaboliczny | Candidate gene<br>Gen kandydacki |
|---|---|---|---|
| **Folate**<br>Folan | **green vegetables, yellow fruits, supplemented food**<br>zielone warzywa, żółte owoce, żywność wzbogacana | **homocysteine remethylation**<br>remetylacja homocysteiny | ***MTR*** |
| **Betaine**<br>Betaina | **beets, spinach**<br>buraki, szpinak | **homocysteine remethylation**<br>remetylacja homocysteiny | ***BHMT1*** |
| **Choline**<br>Cholina | **eggs, red meat, liver, wheat germ**<br>jaja, czerwone mięso, wątróbka, kiełki pszenicy | **homocysteine remethylation**<br>remetylacja homocysremy | ***BHMT1*** |
| **Vitamin U**<br>Witamina U | **Cruciferous vegetables**<br>warzywa krzyżowe | **homocysteine remethylation**<br>remetylacja homocysremy | ***BHMT2*** |
| **Citrulline**<br>Cytrulina | **Cucurbitaceae fruits, birch sap**<br>owoce dyniowate, oskoła | **citrulline/arginine-dependent pathways***<br>szlaki metaboliczne obejmujące przemiany cytruliny/argininy* | ***ASS1, SLC25A13*** |
| **Zinc**<br>Cynk | **red meat, naturally grown produce**<br>czerwone mięso, pokarmy roślinne z upraw ekstensywnych | **transporting zinc to the lumen of organelles or the extracellular space**<br>transport cynku do organelli oraz przestrzeni zewnątrz komórkowej | ***SLC30A5*** |

*i.e. the urea cycle and nitric oxide (NO) homeostasis
*np. cykl mocznikowy i homeostaza tlenku azotu

to be involved in teratogenesis and to contribute to abnormal palatogenesis (reviewed by Hozyasz [37]). Previous biochemical analyses implicated a role in clefting for the antioxidant systems and zinc deficiency in the Polish CL/P population [22, 73, 74]. In spite of this, there was observed no statistically significant associations between maternal polymorphic variants of genes encoding main reactive oxygen species-scavenging enzymes; *CAT*, *GPX1*, mitochondrial superoxide dismutase *MnSOD2*, as well as zinc transporters from the two major unrelated families (SLC30A and SLC39A), and the risk of CL/P-affected pregnancies [24, 33, 87]. However, it has been found that the risk of having a CL/P affected child for the maternal SLC30A5 rs351444 GG genotype compared with the wild type tended to be decreased ($OR_{GGvsCC}$=0.55; 95%CI: 0.26-1.16; p=0.11). Interestingly, haplotype analysis of *SLC30A5* polymorphic variants (rs351444, rs164393, and rs6886492) showed a borderline association between the CTA haplotype and increased risk of clefting (p = 0.051). The exclusion of the investigated *SLC30A5* rs351444, rs164393, rs6886492 and other variants of genes encoding zinc transporters as risk factors of CL/P in the Polish population requires further investigation, which should be performed in larger groups of case and control mothers as well as in CL/P-affected children [33].

## III. Perspective and conclusions

The achievement of a successful reproduction represents one of the fundamental functions of existence. However, every 2 1/2 min, somewhere in the world, a child is born with an orofacial cleft. The focus of this review is on the relationships between a wide range of nutrients and variants of candidate genes or regions and the risk of CL/P in the Polish population. All of these support the need to increase our attention to environment and vulnerable physiology of the embryo. The findings illustrate that the etiology of CL/P is multifactorial and requires the palatogenesis process to be considered on multiple levels and in multiple dimensions. The balance between various environmental, genetic, and epigenetic factors form the complex background of CL/P, which should be carefully explored. Further investigations are needed to identify new gene-environment associations. Most of the reported risk factors in reviewed papers are weak. However, we have to note that multiple risk factors contribute the CL/P etiology, therefore the effect of each factor is rather small. Poor maternal nutritional status leads to many complications, in both the short and long term. The first few weeks of embryo development are particularly sensitive to changes in the maternal environment reflecting changes in the external world. Although most factors appear to explain very little of the population burden of CL/P, maternal nu-

tritional factors do appear to substantially contribute to the complex etiologies of CL/P. There are very few exposures for which the available information is sufficient to make fully evidence-based recommendations regarding the clinical management of teratogenic risks in humans. Nevertheless, physicians must advise pregnant women about identified potential risks [88] and the reviewed papers delivered some new data regarding what constitutes a healthy diet and lifestyle during pregnancy. Experiments with livestock species show that sound nutritional management at key stages in the reproductive process provides an acceptable and effective way to improve the reproductive outcome, not only in terms of the number of offspring born, but also in terms of their physiological well-being and viability [89]. The overarching principles of nutrition are believed to be similar among mammals [89].

If a type of diet has been used for a "long time" without adverse effects being reported, does this represent good evidence that it is safe to use in periconceptional period? Less potent teratogens and unhealthy diet patterns may remain undetected for long periods. Phenytoin was used from 1938 as an antiepileptic drug, while its teratogenic effects were only suggested 30 years after its introduction to the market and supported in 1973 [90]. Very often physicians simply tell women to eat "a healthy diet" and gain appropriate weight during pregnancy. However, to achieve this, they need to show direction to do so properly [91]. Prospective parents should discuss important health behaviors that may affect a pregnancy such as vitamin and micronutrient intake, lifestyle, and occupation with their medical care provider. Until now, conclusive evidence was provided for periconceptional folate and the prevention of neural tube defects [14]. The information from the reviewed research reports regarding the homeostasis of trace elements [22, 25], citrulline [26], and lipid-soluble vitamins [19, 20] is somewhat limited and preliminary. However, it could be useful while preparing some reasonable guidelines for prospective parents, who wish to minimize their chances of having a baby with CL/P. The presented findings affirm for the first time the possible association between deficiency of zinc, vitamin E, and citrulline and the risk of clefting in humans, and call for careful consideration of consumption of foods and supplements that are good sources of these nutrients by women of reproductive age, table II. Circumstantial evidence supports the association of CL/P to the transition from natural unprocessed foods to processed, calorie condensed, Western-type foods [92]. At this time a mixed diet with higher amounts of fruits (including those from the *Cucurbitaceae family*), Crucifereae vegetables, beets varieties, cold-pressed vegetable oils and moderate amounts of red meat and fish seems to be the best recipe for nutritional support for the prevention of malfunctions related to not-optimal nutrition. We should keep in mind, that there is a need for interventional studies, in order to assess potential benefits and to optimize dietary recommendations and thus maternal periconceptional status.

Past and current dietary guidelines have not considered the dramatic differences on the individual's physiological response to changes to nutrient intake [12]. However, these differences in response may greatly affect the efficacy of these recommendations at the individual level. The past few years have witnessed great advances in gene-identification for abnormal palatogenesis [9]. Through variant metabolic pathways and variant growth patterns, genetically susceptible subgroups offer a rich opportunity for research by providing a more sensitive means of identifying expositions that are teratogenic in humans. A healthy diet contains many nutrients working synergistically. Metabolism of folate and cobalamine, as well as betaine/choline and vitamin U, interact at the point homocysteine is converted to methionine. The search of our group [23, 31, 32] for genetic polymorphisms in the homocysteine/folate pathway revealed that polymorphic variants of *MTR*, *BHMT1*, and *BHMT2* are associated with the risk of clefting in the Polish population. *BHMT2*, *BHMT1* and *MTR* convert homocysteine to methionine, but use different methyl donors. It is well known that increased periconceptional intake of folic acid and vitamin $B_{12}$ may reduce the risk of structural malformations [11,14]. However, it remains unclear as to the extent to which SNPs of *MTR*, *BHMT1*, and *BHMT2* contribute to palatogenesis. This newly accumulated knowledge might constitute the basis of new kinds of dietary recommendations. Further work is needed to fully establish the physiological functions and interplay of vitamin U, betaine/choline and their analogues (i.e. trigonelline from tomatoes [93]). Moreover, this observation can raise and support the concept of personalized nutrition aiming at providing targeted dietary advice to women of childbearing age with increased risk of CL/P. From a public health perspective, there is need to create conditions encouraging "healthy choices" of food and to help people make informed decisions within health friendly environments. Highest priority should be given, to the extent possible, to develop nutrigenomics for building a knowledge base for future action. A multidisciplinary approach is the key to ensuring a woman's nutritional goals.

The pathologic mechanisms by which environmental factors influence palatogenesis in humans remain largely unknown. With respect to the findings according *BHMT1*, *BHMT2, MTR, ASS1, SLC25A13, GSTM1, GSTT1*, and *SUMO1* investigation of gene-environment interaction is needed, table 1. Our under-

**Table II.** Key messages
**Tabela II**. Najważniejsze obserwacje i wnioski

**Currently, primary prevention efforts to reduce the occurrence of CL/P are limited to peri-conceptional folic acid use and the avoidance of specific teratogens and exposures, e.g. vitamin A intoxication. Low maternal zinc and α-tocopherol levels and low newborn citrulline level are risk factors for CL/P in the Polish population. The presented findings cali for consideration of an increased consumption of these nutrients. However, triais are needed to assess the potential benefits.**

Profilaktyka pierwotna CL/P w ogólnej populacji jest obecnie w praktyce ograniczona do okołokocepcyjnego przyjmowania przez matkę kwasu foliowego oraz unikania specyficznych teratogenów, np. przedawkowywania witaminy A i jej pochodnych. Małe stężenia cynku i a-tokoferolu u matki oraz małe stężenia cytruliny u noworodka są przypuszczalnie czynnikami ryzyka CL/P w populacji polskiej. Ocena celowości zwiększenia spożycia tych składników wymaga przeprowadzenia badań nad potencjalnymi korzyściami interwencji dietetycznej.

**In the literature, evidence exists for an association between the use of multivitamin supplements and reduced risk of CL/P. However, because of the limitations of the study, the observation regarding multivitamins use as a protective factor constitutes only preliminary evidence in the Polish population which requires further confirmation. The validity of the preventive use of vitamin supplements is supported by the studies on vitamin-related genes as risk factors for abnormal palatogenesis. Dietary and lifestyle profiles should be included in preconception screening programs.**

Obserwacje własne sugerują występowanie w populacji polskiej zależności pomiędzy przyjmowaniem preparatów wielowitaminowych a zmniejszonym ryzykiem CL/P, którą wcześniej udokumentowano w innych populacjach. Użyteczność prewencyjnej suplementacji witaminowej sugerują również wyniki badań nad genami powiązanymi z witaminami jako czynnikami ryzyka zaburzonej palatogenezy. Prekoncepcyjne badania przesiewowe służące zmniejszaniu ryzyka urodzenia dziecka z wadą wrodzoną powinny obejmować analizę diety i wzorca zachowań prozdrowotnych kobiet planujących macierzyństwo.

**Severe deficiency of vitamin $B_{12}$ with increased propionylcarnitine level seems not to be a risk factor for CL/P in the Polish population. There is stil! much knowledge to be gained concerning the regulation of cobalamin status.**

Ciężkie niedobory witaminy $B_{12}$ ze zwiększonymi stężeniami propionylkarnityny nie są przypuszczalnie częstą przyczyną CL/P w populacji polskiej, jednakże wiele zagadnień, dotyczących homeostazy tej witaminy w populacji ryzyka CL/P, nadal wymaga wyjaśnienia.

**Polymorphic variants of MTR and BHMT2 via the maternal genotype and BHMT1 via the embryonic genotype are associated with the risk of c1efting in the Polish population. BHMT2, BHMT1, and MTR convert homocysteine to methionine, but use different methyl donors. This observations highlights the complexity of methyl-group metabolism and may confirm the crucial role of the homocysteine remethylation process during palatogenesis. Further research is needed to fully establish the physiological functions and interplay of folic acid, vitamin U, betaine/choline, and their analogues.**

W populacji polskiej z ryzykiem powstania rozszczepu twarzowej części czaszki związane są polimorficzne warianty genów MTR i BHMT2 matki oraz BHMT1 zarodka. Enzymy BHMT2, BHMT1 i MTR przekształcają homocysteinę w metioninę, przy czym wykorzystują różne źródła grup metylowych. Obserwacje te świadczą o złożoności metabolizmu grup metylowych i stanowią potwierdzenie ważnej roli remetylacji homocysteiny w procesie palatogenezy. Niezbędne jest gruntowne poznanie roli współzależności łączących kwas foliowy, witaminę U, betainę/cholinę oraz ich analogi.

**Polymorphic variants of genes related to arginine/eitrulline metabolism interact on CL/P susceptibility. ASS1, the gene encoding the key enzyme making up the urea cycle, and SLC25A 13 are new candidate genes for structural malformations. Low newborn malonylcarnitine and acetylcarnitine levels seem to be risk factors for CL/P. Determining the precise metabolic impairment that results in CL/P will enable the development of screening programs for decreasing the risk of 'adverse pregnancy outcomes and the design of preventive interventions.**

Polimorficzne warianty genów związanych z metabolizmem argininy/cytruliny współoddziaływują na ryzyko CL/P. ASS1, gen dla głównego enzymu cyklu mocznikowego, oraz SLC25A 13 są nowymi genami kandydackimi malformacji. Małe stężenia karnityny malonylowej oraz acetylkarnityny są przypuszczalnie czynnikami ryzyka CL/P u noworodka. Szczegółowe poznanie zaburzeń metabolicznych towarzyszących CL/P umożliwi opracowanie badań przesiewowych identyfikujących kobiety obciążone zwiększonym ryzykiem zaburzeń zaburzeń w ciąży oraz działań zapobiegawczych.

**Polymorphic variants of IRF6 and the 8q24 loei are strongly involved in the etiology of CL/P in the Polish population. Presented analyses provide moderate evidence that polymorphic variants of SUMO1, GSTM1, and GSTT1 may influence CL/P risk in the Polish population. Genotype frequencies gave evidence for PCYT1A as embryonic, but not maternal CL/P candidate gene. On the other hand, the analysis of maternal SNPs revealed an epistatic interaction among PCYT1A, PEMT, and BHMT2 genes on the risk of having pregnancy affected with an orofaeial cleft. The genetics of palatogenesis is likely a complex interplay between both maternal and fetal susceptibilities. Investigators in future studies should focus on understanding the role of risky polymorphic variants in the development of orofaeial clefts.**

Polimorficzne warianty IRF6 i z lokus 8q24 są silnymi czynnikami ryzyka CL/P w populacji polskiej. Prezentowane analizy wykazały także wpływ SUM01, GSTM1 i GSTT1 na ryzyko CL/P. Różnice w rozkładzie genotypów sugerowały, że PCYT1A jest zarodkowym, ale nie matczynym genem kandydackim. Analiza matczynych polimorfizmów pojedynczych nukleotydów (SNPs) wykazała współoddziaływanie PCYT1A, PEMT i BHMT2 na ryzyko wystąpienia wady rozszczepowej części twarzowej czaszki. Złożone oddziaływania pomiędzy matczyną i zarodkową podatnością współtworzą genetyczne uwarunkowanie CL/P. Zainteresowanie wyjaśnieniem mechanizmów oddziaływania polimorficznych wariantów genów kandydackich na palatogenezę motywuje do dalszych badań.

standing of pathogenesis of CL/P will be enhanced by such studies. There is undoubtedly much work to be done before we fully understand the risk factors contributing to CL/P and it will require breaking many moulds of traditional research and seeking integration of multiple disciplines. At present there is a very limited understanding of the nutrient and non-nutrient-related networks [12]. With the development of new analytical techniques (i.e. MS/MS) and bioinformatics [29, 46, 82], it is likely that future studies will discover new nutritional risk factors and genes, as well as new relationships between genes, pathways, nutritional and other external factors that will elucidate the etiology of CL/P at the indi-

vidual and population level. Presented studies [26, 28, 29, 46] took advantage of the National Newborn Screening Program within Poland, based on MS/MS (secondary data – routinely collected [94]). In several studies of our group epistatic interaction between investigated SNPs on the risk of clefting were tested using the recently developed MDR approach [30-33]. The paper documenting low citrulline levels in newborns with CL/P [27] received some support by independent documentation of interactions between genes related to arginine/citrulline metabolism on CL/P susceptibility [30], table 2.

Among presented studies' the strengths are: 1) That they utilized samples of participants from ethnically homogenous and a mostly omnivorous population; 2) The studies are all region-specific; 3) Adjustment for several potential confounders. The major weakness of presented studies are: 1) The biochemical, genetic, and survey-based studies were not conducted in the same sample of CL/P-affected cases or their mothers. An important area for the further research in the Polish population is investigation of environmental risk factors simultaneously with the investigation of genetic factors; 2) Most studies have examined one nutrient at a time, however, various nutrients may contribute to similar underlying mechanisms and that many nutrients are highly co-related (e.g. dietary methyl group donors); 3) Only one or a few SNPs were tested in each gene. Therefore, failure to find an association for SNPs in some of these genes does not provide conclusive evidence about whether the genes play a role in CL/P; 4) We were not able to evaluate socioeconomic status of participating women in periconceptional period as a confounder, because of the rapid economic transformation in Poland during the last decade. Reports concerning the existence of social class inequalities for congenital anomalies are inconsistent [15,95]; 5) The relatively long periods for maternal recall between periconceptional events of interest and reviewing/blood sampling; 6) Sizes of samples were limited for certain comparisons (e.g. examining individual CL/P phenotypes) [30]. However, in teratology the economical point of view excludes the investigation of large population groups [95]; 6) In the studies devoted to zinc status assessments of the micronutrient were done in blood. Measurement of blood zinc as an indicator of zinc nutritional status is problematic in that only 0.1% of the body's stores are contained in the circulation [33]. Moreover, in interpreting findings on possible associations between risk factors and CL/P, we must remember that such associations from case-controlled studies may be due to factors of interest, but they may also be a result of a chance, bias, and confounding [34]. Different factors could cause the same anomaly when occurring during a specific window of susceptibility. Dosing and duration of the exposure of the fetus to an environmental factor may also be crucial [15, 96].

In summary, many genes and genetic pathways have been implicated in the development of CL/P. Etiological heterogeneity and complex environment-gene interactions may be characteristic of abnormal palatogenesis. The most plausible scenario is that multiple candidate genes will be used to create genetic profiles or scores for CL/P risk, table 2. The diversity of embryological events that contribute to the formation of the facial structures is reflected in the large number of genes known or suspected to be involved in clefting [97]. Some have been determined earlier in foreign populations and confirmed (e.g. *IRF6*, *SUMO1*) or not confirmed (e.g. *FOXE1*, *MSX1*) as CL/P candidate genes in the Polish population. BHMT2 is a new maternal candidate gene with relatively strong evidence. Presented data gave weaker evidence for ASS1 as a CL/P candidate gene. However, keeping in mind results from MDR analysis regarding the *ASS1* rs666174 and *SLC25A13* rs10252573, p values from comparisons of allele and genotype frequencies should not be the only criteria used in assessing candidate genes. CL/P susceptibility loci at 8q24.21 is showing convincing consistency across studies, including our report [27]. Moreover, data provided in presented studies suggest the possible interaction between particular SNPs and metabolic responses to diets, table 1. The more we know about the genetic traits related to CL/P, the easier it will be to access individual risks. Folic acid supplementation in the periconceptional period can largely prevent the occurrence of spina bifida, and there is thus interest in other dietetic interventions that could reduce the prevalence of other structural malformations. In order to prevent CL/P nutrition programs targeted to women before conception should not only emphasize the importance of an adequate intake of folate, but also the importance of a nutrients-rich, balanced diet with an adequate intake of zinc (e.g. from animal sources), antioxidants, amino acids from arginine family (i.e. citrulline from *Cucurbitaceae fruits*), and foods, which positively influence methyl-group homeostasis [98]. Before presented findings on CL/P etiology can be translated into routine public use, they need to be validated by solid scientific evidence.

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