Original Article

Whole blood citrulline concentrations in newborns with non-syndromic oral clefts – a preliminary report

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The amino acid citrulline is poorly represented in food, except for the Cucurbitaceae family of fruits (watermelons, etc.) and sap from birch trees, which have both been used in the treatment of reproductive disorders for centuries. Recently, an interesting observation was published regarding citrulline as a promising candidate biomarker of abnormal embryogenesis. Our study was undertaken to investigate the involvement of newborn citrulline concentrations as potential risk factors for orofacial clefting. We performed a retrospective analysis of citrulline concentrations obtained from the results of a newborn screening program. The study group consists of 52 children with isolated cleft lip with or without cleft palate. One hundred and seven healthy children without congenital anomalies serve as controls. Whole blood citrulline levels were measured using tandem mass spectrometry. Two cut-off points of citrulline concentrations were accepted and three groups of participants were analysed: those with (1) low concentrations (<8 μmol/L), (2) medium concentrations (8 - 16 μmol/L), and (3) high concentrations (>16 μmol/L). Low citrulline levels were significantly more frequent in patients with clefts than in children without congenital anomalies. In the control group, a high level of citrulline was observed nearly two times more often than in affected children (p=0.03). The presented findings, confirming a possible association between newborn citrulline status and the risk of an orofacial cleft, call for further studies in this area and consideration of increased consumption of foods rich in citrulline (or supplements) by women of childbearing age.

Key Words: cleft palate, citrulline, neonate, metabolomics, tandem mass spectrometry

INTRODUCTION

Cleft lip with or without cleft palate (CLP) is one of the most common congenital abnormalities in humans. Approximately one in 500 to 3000 children is born with an orofacial cleft. The presence of this abnormality has severe consequences on both physical and psychological development and imposes a substantial economic and social burden. The etiology of CLP is complex and associated with both genetic and environmental factors. So far, research on the association between nutritional status of infants and abnormal palatogenesis has mainly been focused on vitamins and trace elements.[1-3] However, a trend towards risk reduction of orofacial clefts by increased maternal consumption of certain amino acids was recently demonstrated by Shaw et al.[4]

Human embryonic stem cells (HESC) have the potential of constituting an in vitro model for early human developmental disorders. The identification of small molecules from HESC using metabolomics may serve to determine biochemical pathways that participate in abnormal embryogenesis and provide candidate biomarkers for further studies.[5,6] Valproate exposure in early pregnancy is a risk factor for both neural tube defects and orofacial clefts in offspring.[7] Recently, Cezar et al.[5] reported observations of the effects of valproate treatment on HESC differentiated to neural precursors. Of particular interest is their finding that a small molecule with a mass spectral match to citrulline was specifically upregulated. The investigators stated that citrulline may serve as a candidate biomarker of abnormal embryogenesis and proposed to examine selected small molecules from the HESC cell metabolome in infants with congenital anomalies.[5]

Citrulline is a water-soluble, non-essential amino acid regarded mainly as a metabolic intermediate in the urea cycle. However, it also plays an important role in the metabolism and regulation of nitric oxide. Citrulline produced in the liver is compartmentalized as an intermediate in the urea cycle and the conversion of glutamine in enterocytes is a major source of the circulating amino acid. Citrulline is poorly represented in food except in cucurbitaceus fruits and sap from birch trees.[8] The name of the amino acid is derived from *Citrullus vulgaris*, commonly known as the watermelon, which may contain up to 3.5g of citrulline per kg.[9] The sap of birch (*Betula pendula*, *Betula pubescens*) was valued by prechristian

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Manuscript received 9 July 2009. Initial review completed 17 December 2009. Revision accepted 18 January 2010.

Slavic nations as God's drink of life. In Eastern European and Northern Asian ethnomedicine it is a popular remedy for a wide range of female health problems, e.g. for improving fertility.[10,11] Temple food in Korean Buddhism, the consumption of which results in better general health, includes several fruits from the Cucurbitaceae family.[12] Watermelon consumption may also play a role in optimizing reproductive health.[13]

Amino acid analysis of dried whole blood spots using tandem mass spectrometry (MS/MS) to detect inherited metabolism disorders is one of the most important advancements in neonatal screening.[14] The experiences from MS/MS screening provide important insights about the diagnostic potential of metabolome analyses.[15] Dried blood spot amino acid MS/MS analysis is also used for monitoring patients with diseases involving citrulline metabolism disorders due to a reduction of enterocytes mass.[16-18]

Many environmental risk factors have been linked to CLP formation, though there have been few consistent findings. The purpose of our study was to analyze citrulline levels in newborns with CLP and in healthy controls.

MATERIALS AND METHODS

Study population

Patients with isolated cleft lip with or without cleft palate attending our institution and unrelated healthy children of similar age – the patients of three local primary care pediatricians – were considered for inclusion in the study. Inclusion criteria were as follows: (1) singleton pregnancy, (2) gestational age at delivery ≥ 36 weeks and/or birth weight >2000 g, which have long been recognized as important determinants of newborn health, (3) delivery in the years 2004-2007 in hospitals located within the area covered by the MS/MS Newborn Screening Program provided by our institution. Most newborn blood samples were collected 2-4 days after birth.

Case eligibility was determined from detailed medical records. Finally, we performed a retrospective analysis of newborn MS/MS citrulline screening results of 52 children with CLP and 107 healthy controls without congenital anomalies. Slightly more case newborns were males (67%) compared with the control group (62%), p >0.05. Mean gestational age at delivery of cases and controls were the same (39 weeks). Birth weights of CLP newborns were slightly lower than that in non-affected children (3390 g vs. 3540 g, p=0.05). All mothers were white, healthy and omnivorous. It was assumed that none took supplements with citrulline, because such products have not been dispensed in Poland.

The study protocol were approved by the Ethics Committee at Institute of Mother and Child in Warsaw.

Mass spectrometry

Two 3.5 mm dried blood spots (equivalent to 7.5 µL of blood) were punched into a 96 well microtiter plate. Methanolic internal citrulline standard solution (100 µL) was added with an 8 chanel pipette. The microtiter plate was shaken over a 20-min extraction. The extract was transferred to a second microtiter plate and dried. The residue was butylated with butanol-HCl at 65°C for 20 min., then dried and dissolved in 50 µL of methanol water 50:50 mixture with 0.02% formic acid. Samples were analysed in tandem mass spectrometer (SCIEX Api 2000) equipped with a liquid chromatography autosampler. The working range was 1-300 µmol/L of citrulline.

Statistical methods

Statistical methods used for the analysis of citrulline concentrations as a continuous variables included the Spearman correlation analysis and the Mann-Whitney test. The chi-square test was used to investigate the relationship between categorical parameters. Citrulline level cut-off points were established using likelihood ratio values (the ratio of the maximum probability of a result using two different hypotheses). For the multivariable analysis, a logistic regression model was used. Statistical significance was interpreted as p values < 0.05. All statistical analyses were performed using the SPSS version 12.0.1 for Windows (SPSS Polska Sp. z o.o., Cracow, Poland).

RESULTS

The median (interquartile range, IQR) concentrations of whole blood citrulline in newborns with orofacial cleft and controls averaged 12.5 (10.7-15.7) µmol/L and 14.5 (11.1-17.8) µmol/L, respectively (Figure 1). The citrulline/arginine homeostasis allows a proper supply of arginine for the whole body. Correlation analyses of the whole blood amino acids concentrations revealed significant correlations between citrulline and arginine in cases and

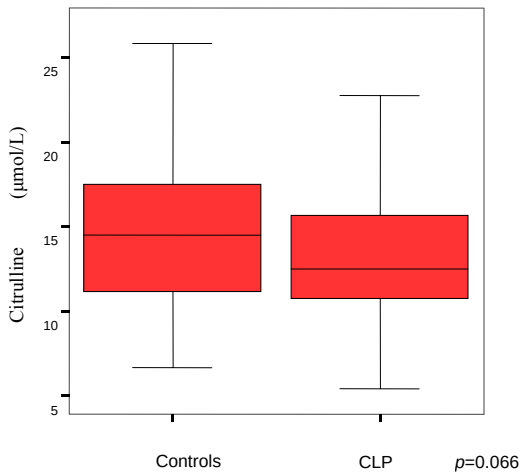

Figure 1. Diagram presenting median levels of citrulline in newborns with CLP and controls.

controls; r=0.614 (p <0.001) and r=0.413 (p <0.001), respectively.

The univariable analysis

The occurrence of orofacial cleft in newborns had a tendency of being related to citrulline levels, p=0.066 in the Mann-Whitney test. The likelihood ratio test indicated two citrulline level cut-off points: 8 μmol/L and 16 μmol/L. We therefore analysed three groups of individuals: with citrulline levels (1) <8 μmol/L (n=8), (2) between 8 and 16 μmol/L (n=96), and (3) >16 μmol/L (n=55). Figure 2 shows that in patients with CLP, low citrulline levels were three times more predominant 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 CLP, 43/107 (40.2%) vs. 12/52 (23.1%), p=0.03. There were no significant correlations between citrulline levels and clinical variables such as birth weight or gestational age at delivery.

The multivariable analysis

The logistic regression model simultaneously adjusted for birth weight and the three categories of citrulline concentrations confirmed that citrulline levels are important risk factors for the occurrence of CLP (p=0.034). Low citrulline levels are associated with a high risk of an infant being born with an orofacial cleft (odds ratio (OR) 6.0; 95%C.I.: 1.2-29.2). The risk is reduced when citrulline levels are between 8-16 μmol/L (OR 2.2; 95%C.I.: 1.0-4.9). The risk is lowest when the level of citrulline in routine neonatal screening is above 16 μmol/L (Table 1).

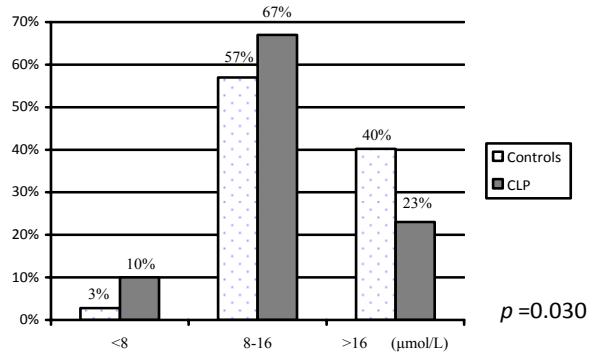

Figure 2. Results of newborn citrulline screening in children with CLP and controls.

Table 1. Associations of the relative risk of bearing a CLP-affected child with low or high neonatal citrulline levels in the multiple logistic regression model

	Odds Ratio	95% CI	p value
Birth weight, g			
>3000 vs. ≤3000	0.562	(0.21-1.50)	0.250
Citrulline, μmol/L			
> 16	1.00		
8 -16	2.25	(1.02-4.95)	0.043
< 8	6.01	(1.24-29.2)	0.026

DISCUSSION

Associations between maternal drugs and chemicals exposure, diabetes mellitus, smoking, hyperhomocysteinaemia, viral infections with hyperthermia in the first trimester and an increased risk of having a child with CLP were reported.[2,3] These risk factors are known to affect both antioxidant systems as well as nitric oxide homeostasis.[17] Citrulline and citrulline-dependent metabolic pathways appear to play a more important role in early mammalian embryo development than previously believed.[19-21] Citrulline is the third most abundant amino acid in ovine allantoic fluid in early gestation.[20] As a neutral amino acid, which does not influence *pH* balance when concentrated, citrulline may serve as an antioxidant and an efficient reservoir of precursors for arginine synthesis.[22,23] In recent studies, a new concept concerning citrulline has emerged, presenting the amino acid as an important regulator of nitrogen homeostasis.[24] Nitric oxide metabolism disorders may disturb oocyte maturation, implantation, embryonic vasculogenesis, and vascular function.[25] The expression of methionine synthase is regulated by several factors including nitrous oxide. This enzyme catalyzes the remethylation of homocysteine to methionine – the only reaction in humans in which folate and vitamin B_{12} are co-partcipants. A common polymorphic variant of the gene for methionine synthase (*MTR* A2756G), as well as deficiencies of folate and vitamin B_{12}, were found to be positively associated with abnormal palatogenesis.[26] Moreover, in mice, methionine supplementation has been shown to ameliorate valproate-induced abnormalities during embryogenesis,[27] but the influence of this intervention on citrulline regulation in HESC has not yet been studied. In adults, it is estimated that citrulline contributes to 10% of the whole body arginine production.[28] Oral arginine supplementation improves endometrial receptivity and pregnancy rate, and contributes to an increased citrulline concentration in follicular fluid in humans.[29,30]

Our results provide unprecedented, but weak (p=0.03), evidence that a low whole blood citrulline level (<8 μmol/L) in the newborn is associated with an increased risk of an orofacial cleft. On the other hand, a high citrulline level (>16 μmol/L) is probably associated with a decreased risk of CLP. However, the median citrulline level in newborns with CLP in comparison to unaffected children only tended to be slightly lower and a borderline association, at most (p=0.07). This may have been the result of a relatively small number of analyzed individuals.

There are no kinetic studies of citrulline in newborns.[17,24] In adults blood citrulline levels can be increased through an intake of food rich in citrulline.[31,32] It is currently impossible to decipher whether lower citrulline levels in some newborns with a cleft were caused by a low intake of this amino acid by the mother, decreased endogenic synthesis or an increased utilization of citrulline. It is also impossible to conclude whether citrulline directly affects palatogenesis or whether citrulline metabolism disorders are the cause of CLP in humans. This matter requires further clarification. It therefore seems reasonable to test mutated genes involved in citrulline metabolism as CLP candidate genes.

Several nutritional treatments for pregnancy-related conditions appear promising and recent trends indicate the increased use of botanicals.[29] Our study, as well as the observations of Westphal et al.[29] and Battaglia et al,[30] suggest that offspring may likely benefit from an intake of plant food rich in citrulline by the mother. Intrauterine development is influenced by maternal behavior and genetic attributes. Mothers with the highest scores of the Western diet pattern, which is low in fruits, demonstrated a two-fold higher risk of having a child with cleft lip with or without cleft palate.[33,34] The study of Vujkovic et al.[34] provided no detailed data on amino acid consumption, however, it can be speculated that an intake of plant food rich in citrulline seems to contribute to the prevention of clefts. Maternal diabetes is a well-known risk factor for orofacial clefts.[35] Both diabetes and exposure to valproate influence nitric oxide metabolism and antioxidant defense mechanisms.[7] Experimental studies have shown that dietary supplementation with watermelon juice ameliorates the metabolic syndrome.[36]

So far, very few studies have investigated maternal and infant amino acid homeostasis in relation to orofacial clefts.[3,37] Our novel finding raises important questions regarding human fetal metabolism of citrulline, which is traditionally classified as a non-essential amino acid. In animal husbandry, the use of supplements containing amino acids from the arginine family for reproduction and pregnancy is widely recognized.[38] The therapeutic use of amino acids therefore presents medical professionals with an interesting alternative.[39] Amino acids are not stored for long periods of time and pregnancy involves specific amino acid requirements for maternal maintenance, growth of the fetal tissue and placenta, and growth of mammary glands. The challenge for medical professionals has been a lack of evidence regarding which nutrients and foods are most effective in fulfilling these requirements.

It remains unclear whether increased maternal citrulline intake may be causally associated with reduced risk of CLP. Collecting samples such as dried blood spots is the simplest method, feasible to carry out in any doctor's clinic, even in those not equipped with a centrifuge. No single study is likely to be definitive and the facilitated collection of specimens may encourage other investigators dealing with orofacial clefts. The confirmation of our observation requires further analyses of citrulline and other amino acids from the arginine family, which should be performed in larger groups of affected newborns and controls as well as their mothers. The identification of environmental risk factors holds the promise of determining preventive strategies.

Our study has some limitations to consider. Firstly, a small number of cases were examined. Secondly, the citrulline level assessments were carried out after delivery and only in newborns which may have lowered the possibility of finding teratologically pertinent feto-maternal changes. Cord blood is of limited value in detecting inherited metabolic diseases.[40] Since we performed the retrospective analysis of results from routine MS/MS newborn screening, the blood samples were collected 2-4 days after birth. Our study design also has limitations because of its retrospective nature. We lacked the ability to access additional information on newborn feeding, maternal intake of nutrients, medicine and supplement use, smoking status, weight gain during pregnancy, as well as maternal education. Adequate nutrition of the mother at the time of conception and in early pregnancy appears to be important for normal organogenesis.[4,33-35] Cleft lip and palate develops between the fourth and tenth weeks of gestation and collection of samples during this time would require tens of thousands of participating mothers in a prospective study.

This study also displays some notable strengths. The citrulline assessment results used in our study were obtained from a population-based MS/MS Newborn Screening Program, and we were able to distinguish between different phenotypic cleft types so that only isolated CLP cases were included. Cases and healthy controls without congenital anomalies were under our observation for at least one year. The ethnically homogeneous population under investigation was from an area where preconceptional supplement use is uncommon.

CONCLUSION

This study demonstrates for the first time that citrulline, identified using metabolomic methods is a candidate biomarker of abnormal embryogenesis. It is highly represented only in some plant foods that have been used in the treatment of reproductive disorders for centuries, and may be involved in the etiology of orofacial clefts.

Proper nutrition is a prerequisite for optimizing fertility and childbearing potential. However, it is not clear which nutrients are key in CLP prevention. The presented findings, affirming the possible association between newborn citrulline status and the risk of orofacial cleft, call for further studies in this area and consideration of an increased consumption of foods rich in citrulline (or supplements) by women of childbearing age.

AUTHOR DISCLOSURES

The authors do not have any commercial affiliation that would affect the integrity of this manuscript. No substantive assistance was received in preparation of the manuscript.

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Original Article

Whole blood citrulline concentrations in newborns with nonsyndromic oral clefts – a preliminary report

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非症候型唇顎裂的新生兒之全血瓜胺酸濃度—初步報告

瓜胺酸是食物中少量存在的胺基酸，只有葫蘆科的水果(例如西瓜等)以及樺樹的樹液除外，而這些都已被用來治療生殖系統失調數個世紀了。最近發表了一項關於瓜胺酸可能做為胚胎發育異常的生物標記的觀察，受到注意。我們的研究是調查新生兒的瓜胺酸濃度是否為顏面唇顎裂的潛在風險因子。由一項新生兒篩檢計劃中取得瓜胺酸濃度來進行回溯性分析。研究族群中包括 52 位有單純性唇裂伴隨有或無顎裂的小孩，107 位無先天性異常的健康小孩作為對照組。全血瓜胺酸濃度是利用串聯式質譜儀進行測量。以瓜胺酸濃度的兩個切點，分成下列三個組別進行分析：(1)低濃度(小於 8 μmol/L) (2)中等濃度(8–16 μmol/L) (3)高濃度(大於 16 μmol/L)。低瓜胺酸濃度在先天性唇顎裂的小孩，比無異常的小孩更常見。對照組小孩具有高瓜胺酸濃度的比例為病例組的兩倍。本文研究結果，證實新生兒瓜胺酸濃度的狀態與先天性唇顎裂的危險性之間的可能關聯。呼籲需要此領域的進一步研究以及育齡婦女增加富含瓜胺酸的食物(或補充劑)攝取。

關鍵字：唇顎裂、瓜胺酸、新生兒、代謝組學、串聯式質譜儀