ORIGINAL ARTICLE

# Cystathionine β-synthase genetic variant rs2124459 is associated with a reduced risk of cleft palate in French and Belgian populations

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## ABSTRACT

**Background** Orofacial cleft (OFC) is the most prevalent craniofacial birth defect. Genes involved in one-carbon, folate and vitamin $B_{12}$ metabolisms have been associated with OFC but no study performed a concomitant assessment on genes involved in these three pathways.

**Objective** We looked for potential genetic variants associated with OFC using an exhaustive gene panel of one-carbon metabolism.

**Methods** We performed a case–control discovery study on children with OFC (236 cases, 145 controls) and their related mothers (186 cases, 127 controls). We performed a replication study on the top significant genetic variant in an independent group from Belgium (248 cases, 225 controls).

**Results** In the discovery study on 'mothers', the *CBS* locus reached array-wide significance ($p=9.13\times10^{-6}$; Bonferroni $p=4.77\times10^{-3}$; OR 0.47 (0.33 to 0.66)) among the 519 haplotypes tested for their association with OFC risk. Within the *CBS* haplotype block (rs2124459, rs6586282, rs4920037, rs234705, rs234709), the rs2124459 was the most significantly associated with a reduced risk of OFC ($p=1.77\times10^{-4}$; Bonferroni $p=2.00\times10^{-2}$; OR 0.53 (0.38 to 0.74), minor allele). The rs2124459 was associated with a reduced risk of cleft palate (CP) ($p=6.78\times10^{-5}$; Bonferroni $p=7.80\times10^{-3}$; OR 0.40 (0.25 to 0.63)). In the 'children' group, the rs2124459 was associated with a reduced risk of CP (p=0.02; OR 0.61 (0.40 to 0.93), minor allele). The association between rs2124459 and reduced risk of CP was replicated in an independent children population from Belgium (p=0.02; OR 0.64 (0.44 to 0.93), minor allele).

**Conclusions** The *CBS* rs2124459 was associated with a reduced risk of CP in both French and Belgian populations. These results highlight the prominent involvement of the vitamin B6-dependent transsulfuration pathway of homocysteine in OFC risk and the interest for evaluating vitamin B6 status in further population studies.

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## INTRODUCTION

Orofacial cleft (OFC) is the more prevalent craniofacial birth defect in the world.[1] Three clinical OFC phenotypes are individualised depending on anatomical and embryological features: (1) cleft lip (CL), corresponding to the cleft forward the maxilla foramen totally or not totally; (2) cleft palate (CP), behind the maxilla foramen, totally or not totally; and (3) CL and cleft lip palate (CLP), with both anomalies.[2] In a worldwide survey performed by the WHO that gathered data from three registries (http://www.icbd.org, http://www.eurocat.ulster.ac.uk and http://www.nbdpn.org) and coordinated by the International Centre of Birth Defects, the global incidence rate of OFC was 15 per 10 000 births (95% CI 15 to 16).[3] Interestingly, there was a high variability of OFC incidence rates across the different geographical regions. OFC has a higher prevalence in both Asian populations and Native Americans and a lower prevalence in the African population compared with Caucasian populations.[4] The relatively low prevalence of OFC in South Africa (2.89 per 10.000 births; 95% CI 1.76 to 4.66) has to be compared with the sixfold higher incidence observed in Western Europe (12.10 per 10 000 births; 95% CI 11.10 to 13.18).[5]

The one-carbon metabolism (OCM) is involved in three essential roles in DNA synthesis homeostasis: purines synthesis, thymidine synthesis and DNA/RNA methylation, using S-adenosyl methionine as methyl donor (for details, see figure 1 in ref. 6). The deficiency in folate and vitamin $B_{12}$ impairs these pathways.[7] Folate supplementation during the periconceptional period reduces the risk of OFC.[8] Women who used multivitamins containing folic acid during the periconceptional period have a 25–50% reduction in risk of OFC occurrence compared with non-user women.[9 10] In a prospective study, the OFC recurrence decreased from 7.4% to 4.1% in women who received folate-containing multivitamins supplements compared with non-supplemented women.[11] Animal models showed a causal relationship between folate and/or vitamin $B_{12}$ status and the risk of OFC in rats.[12] In both human and animals studies, the intake of drugs that impair OCM, like methotrexate or carbamazepine, has been shown to be associated with an increased risk of neural tube defects,[13 14] but data regarding the risk of OFC are less clear.[15] Altogether, these data highlight the major role of OCM in OFC pathogenesis.

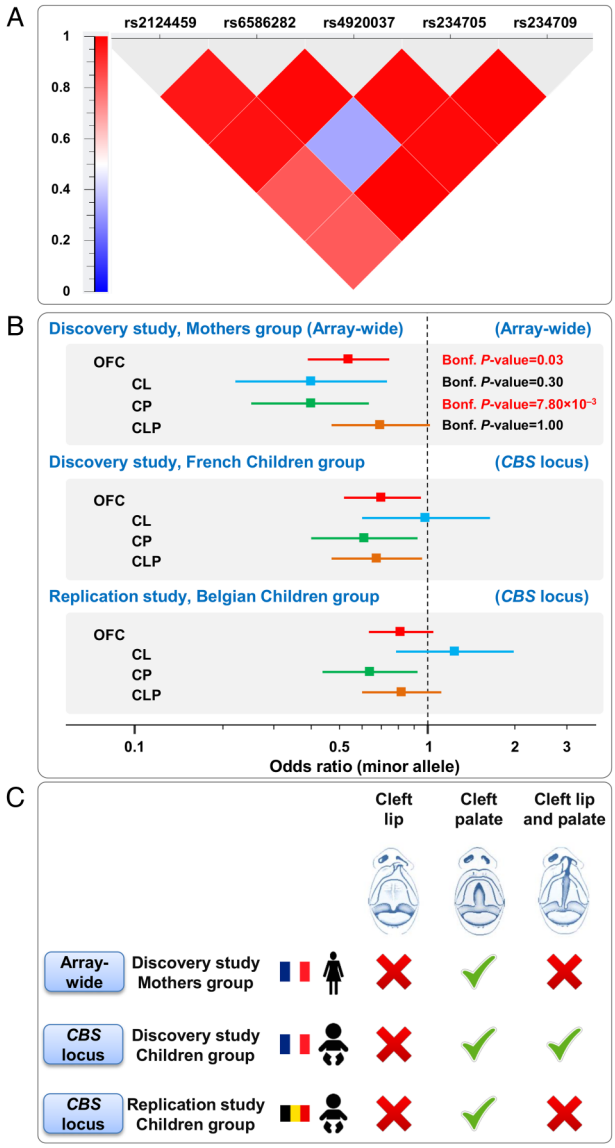


**Figure 1 (available in colour online)** (A) Genomic context and LD plot of the five genetic variants of the *CBS* locus (rs2124459, rs6586282, rs4920037, rs234705, rs234709) forming the haplotype block significantly associated with orofacial cleft (OFC) risk. (B) Forest plot showing the association between the top significant variant on *CBS* (rs2124459) and orofacial cleft risk in the discovery study (mother and children groups) and the replication study. (C) Summary of the study results; the green check mark represents a significant genetic association with rs2124459; the red cross means there is no significant genetic association with rs2124459. Bonf., Bonferroni; CL, cleft lip; CLP, cleft lip palate; CP, cleft palate.

It has been demonstrated that OFCs have a genetic substratum, with as much as 55% of heritability in recent genomewide association studies.[16 17] It is well known that genes that regulate folate metabolism and OCM are strongly involved in birth defects, particularly in the pathogenesis of defects that potentially share common pathogenetic mechanisms with OFC, including neural tube defects.[18] Despite this evidence, studies that have evaluated the effect of OCM-related genetic determinants on the risk of non-syndromic OFC occurrence or recurrence have not produced conclusive data.[19]

Through an exhaustive review of the literature, we identified 19 genes that could be involved in the primary risk OFC occurrence (see online supplementary table S1). Among these genes, most of the studies have been focused on *MTHFR*, with a particular interest on rs1801133 (c.665C>T).[20] This single-nucleotide polymorphism leads to the synthesis of a thermolabile form of the MTHFR protein, which catalyses the reduction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the methyl donor needed for the remethylation of homocysteine (Hcy) to methionine.[6] Other studies have devoted their interest to the *CBS* gene and the transsulfuration pathway of Hcy.[21] However, most of the case–control studies were not followed by replication studies (see online supplementary table S1). In addition, the absence of convincing data is also explained by the absence of any systematic evaluation of OFC risk through a fine mapping association study on OCM gene variants.

To address this issue, we assessed the association between genetic variants on OCM genes and OFC risk in thoroughly phenotyped children with OFC and their mothers compared with matched controls, using an in-house designed array, and we replicated the most relevant association in another sample population of OFC children. We found that a polymorphism in the *CBS* gene, involved in the transsulfuration pathway of OCM, was associated with a protective effect on OFC risk in both populations.

## PATIENTS AND METHODS

### Study design

We performed a discovery study in OFC children and their mothers compared with matched controls and replicated the top significant genetic variant identified in the discovery step in an independent group of OFC children from Belgium.

### Primary and secondary outcomes

The primary outcome of the study was to look for potential genetic variants associated with the primary risk of OFC occurrence using a comprehensive in-house designed OCM array. The secondary outcomes were to look for potential genetic variants potentially associated with the three OFC clinical phenotypes, namely CL, CP and CLP.

### The FePA discovery study

The FePA study (*Fentes Palatines et Anomalies du métabolisme des monocarbones*, in French; OFC and abnormalities of one-carbon metabolism, in English; *Hospital Program for Clinical Research* PHRC, ID-RCB, AFSSAPS, No. 2008-A00924-51) included prospectively recruited children (236 cases, 145 controls) and their mothers (186 cases, 127 controls). Children cases and their mothers were enrolled in two departments of maxillofacial surgery (University Hospital of Nancy and University Hospital of Tours, France) and were matched to healthy controls recruited in the same centres. We did not include mothers and children who received any medication that could influence vitamin $B_{12}$, folate or Hcy metabolism. All children or mothers with clinical sign of 22q11 deletion or other genetic syndromes were not included in the study. The Nancy University Hospital ethics committee approved the study protocol, and all the subjects included in the FePA study gave a written consent. No specific venous blood collection was performed among children controls as requested by the Nancy University Hospital ethics committee. In both mothers and children groups, the following data were collected at the time of inclusion: cleft phenotype; comorbidity; birth weight; history of mother's miscarriage; familial history of cleft, neural tube defect, conotruncal heart defects, age of the mother and of the

father at the conception; date of birth; and concomitant medications. For blood sampling, phlebotomy was performed and fasting venous blood was collected in EDTA-containing tubes. Samples were centrifuged immediately at 2500 *g* for 15 min at room temperature. Aliquots were stored at −70°C until analysis. For children controls, cord blood was used.

### Replication study

In the replication study, 248 children with OFC that were matched with 225 healthy children prospectively enrolled during the same time period in the department of Laboratory of Human Molecular Genetics, de Duve Institute, Université Catholique de Louvain, Brussels, Belgium.

### Analyses on genomic DNA

DNA was isolated from a lymphocyte-enriched fraction of whole blood with NUCLEON BACC3 for extraction of genomic DNA kit (Amersham Pharmacia Biotech, Milan, Italy). Genotyping of polymorphisms involved in OCM was carried out on Illumina VeraCode BeadXpress platform (Illumina, San Diego, California) using a plex of 384 genetic variants (OCM array), which was designed in the laboratory unit INSERM_U954 of the National Institute of Health and Medical Research by two investigators (LG and J-LG). In a first step, all genetic variants included in the 'OCM array' were selected according to a comprehensive literature review with previously defined search strategy by using NCBI (http://www.ncbi.nlm.nih.gov/guide), HapMap (http://hapmap.ncbi.nlm.nih.gov) and Ensembl (http://www.ensembl.org/index.html) databases. Genetic variants were also selected if they were associated with a change in the function, the regulation, the splicing site or the turnover of the transcript or if they were associated with a modification of the phenotype of OCM enzymes (see online supplementary table S2). In a second step, using the Illumina Assay Design Tool (http://www.Illumina.com) we performed an in silico validation of all identified genetic variants in order to determine their suitability for genotyping with the GoldenGate assay. The assessment was conducted based on the following criteria: (1) Illumina's in-house criteria through which variants were assigned a designability score of 1 (highly designable), 0.5 (moderately designable) or 0 (low designability) and (2) the 60 bp limitation rule because a given variant cannot be closer than 60 bp to another one on the oligonucleotide pool assay. Variants with zero designability score were discarded and the final selection of the 384 variants was sent to Illumina for the synthesis of the GoldenGate genotyping assay. Online supplementary table S3 reports the 384 genetic variants included in the 'OCM array' according to the above-mentioned strategy. Genotyping was performed using BeadXpress System (Illumina, Paris, France). All data were analysed by BeadStudio GT Module V.3.2.

We genotyped the *CBS* rs2124459 in the replication study using fluorescence resonance energy transfer real-time PCR (TIB MOLBIOL, Berlin, Germany) on LightCycler 480 (Roche Applied System). It has been shown that the deleterious effect of the *CBS* variant rs5742905 (c.833T>C, p.Ile278Thr) was antagonised by the insertion c.844_845ins68 through a skipping mechanism when in *cis* position.[22] Consequently, we have specifically assessed the occurrence of these two *CBS* variants in *cis* position through restriction fragment length polymorphism technique (RFLP) as previously described in a subset of 237 children and 262 mothers from the derivation study.[23] Separation of PCR-RFLP products was assessed using migration on 1.5% agarose gel.

### Biological markers of OCM

We measured folate, vitamin $B_{12}$, Hcy, methylmalonic acid (MMA), haptocorrin (ApoHC), holotranscobalamin (holoTC) and apotranscobalamin 2 (ApoTC2) in plasma. All specimens were tested in a single laboratory (Department of Molecular Medicine and Personalized Therapeutics, Division of Biochemistry, University Hospital of Nancy, France). Hcy and MMA were determined by ultra performance liquid chromatography (UPLC)-tandem mass spectrometry procedure as previously published, with an Acquity UPLC BEH C18 column (1.7 µm, 2.1×50 mm) (Waters Corporation, Versailles, France).[24] Plasma vitamin $B_{12}$ and folate concentrations were assayed by using a vitamin $B_{12}$ and folate immunoassay kit by means of automated chemiluminescence (Chiron Diagnostics ASC:180 Automated chemiluminescence Systems; East Walpole, Massachusetts, USA). The intra-assay coefficients of variation for vitamin $B_{12}$, folate, Hcy and methylmalonate were 2.8%, 5.1%, 4.5% and 7.3%, respectively. ApoTC2 and ApoHC blood concentrations were measured by differential precipitation and radioassay on Cobra II auto-gamma (UB12BC-totale program). HoloTC levels were performed by Microparticle Enzymatic Immuno Assay (AxSYM HoloTC, Abbot, USA).

### Statistical analysis

All quantitative variables are described as medians and percentiles (IQR, 25th–75th percentile). All proportions and genotype frequencies are expressed as percentages with 95% CIs. Quality controls were performed before genetic association analysis on the 'mothers group' data set. All genetic variants with a call rate <0.95, a minor allele frequency <0.01 and those with a deviation from Hardy-Weinberg equilibrium ($p<10^{-4}$) were removed prior to analysis. In the derivation study on mothers group, we performed haplotype association tests approach using a moving window with a dynamic width of 10 kb. Association tests were calculated per haplotype using $\chi^2$ test and multiple testing correction was carried out using Bonferroni correction. In each comparison, subjects with the studied haplotype were opposed to those with other haplotypes. Effect size was reported using OR and 95% CI. Haplotype estimation method was based on the following criteria: (1) display threshold: 0.01 (0 blocks skipped because of too many or too few possible haplotypes), (2) estimation method: expectation/maximisation (EM) algorithm, (3) maximum EM iterations: 50 and (4) EM convergence tolerance: 0.0001.[25] The comparison of genetic variant frequencies between cases and controls was carried out using Fisher's exact test for the allelic model with Bonferroni method for multiple testing correction. LD pairwise analysis was performed using a matrix output for both the EM algorithm and composite haplotype method and D′ value.[26] The comparison of OCM markers' concentration across genotypes was performed with Kruskal-Wallis test and correlation trend test. All the reported p values were two-sided, and p values <0.05 were considered statistically significant. Statistical analyses were performed using SNP & Variation Suite V.8.4.4 (Golden Helix, Bozeman, Montana, USA, http://www.goldenhelix.com) and MedCalc software, V.11.4.4 (MedCalc Software, Mariakerke, Belgium).

## RESULTS

The baseline characteristics of the mothers and controls included in the study are summarised in online supplementary tables S4 and S5. In the discovery study, children with CL, CP and CLP represented 16.7%, 53.0% and 30.3%, respectively.

**Table 1** Genetic association between the *CBS* variant rs2124459 and orofacial cleft (OFC) risk in the discovery study (mother and children groups) and the replication study

| Study | Fisher's exact p value | Fisher's exact Bonferroni p value | OR (minor allele) | 95% CI | MAF, cases | MAF, controls |
|---|---|---|---|---|---|---|
| *Discovery study, mothers group, France* | | | | | | |
| OFC | $2.44\times10^{-4}$ | 0.03* | 0.54 | 0.39 to 0.74 | 0.37 | 0.52 |
| CL vs controls | $2.67\times10^{-3}$ | 0.30* | 0.40 | 0.22 to 0.73 | 0.31 | 0.52 |
| CP vs controls | $6.78\times10^{-5}$ | $7.80\times10^{-3}$* | 0.40 | 0.25 to 0.63 | 0.31 | 0.52 |
| CLP vs controls | $7.63\times10^{-2}$ | 1.00* | 0.69 | 0.47 to 1.02 | 0.43 | 0.52 |
| *Discovery study, children group, France* | | | | | | |
| OFC | 0.02 | † | 0.70 | 0.52 to 0.95 | 0.39 | 0.48 |
| CL vs controls | 1.00 | † | 0.99 | 0.60 to 1.64 | 0.47 | 0.48 |
| CP vs controls | 0.02 | † | 0.61 | 0.40 to 0.93 | 0.36 | 0.48 |
| CLP vs controls | 0.03 | † | 0.67 | 0.47 to 0.96 | 0.38 | 0.48 |
| *Replication study, children group, Belgium* | | | | | | |
| OFC (exploratory) | 0.13 | † | 0.81 | 0.63 to 1.05 | 0.40 | 0.45 |
| CL vs controls (exploratory) | 0.40 | † | 1.24 | 0.78 to 1.97 | 0.50 | 0.45 |
| CP vs controls (primary end point) | 0.02 | † | 0.64 | 0.44 to 0.93 | 0.34 | 0.45 |
| CLP vs controls (exploratory) | 0.20 | † | 0.82 | 0.60 to 1.12 | 0.40 | 0.45 |

*Genetic association study performed on array-wide basis.
†Genetic association study restricted to the *CBS* locus.
CL, cleft lip; CLP, cleft lip and palate; CP, cleft palate; MAF, minor allele frequency.

In the replication study from Belgium, the same figures were 16.9%, 31.9% and 51.2%, respectively. Based on genetic data from the discovery cohort (mothers group), among the 384 genetic variants present in the OCM array, 246 were excluded on the basis of their minor allele frequency (n=193; threshold<0.01) and/or quality control checks (call rate <0.95 and/or Hardy-Weinberg equilibrium p value in controls $<10^{-4}$, n=53), leaving 138 in the final analysis (see online supplementary table S6). The 138 variants corresponded to 519 haplotype blocks (see online supplementary table S7).

## Predictors of OFC risk in the discovery study

### Mothers group

Among 519 haplotypes tested for their association with the risk of OFC, only one reached array-wide significance and was located in the *CBS* locus (cystathionine-β-synthase; gene ID: 875) in the chromosome 21q22.3. This *CBS* haplotype '*Ddddd*' (*D*: minor allele; *d*: major allele) included five intronic variants (rs2124459, rs6586282, rs4920037, rs234705, rs234709, ordered according to their genomic position) and was associated with a reduced risk of OFC ($\chi^2$ $p=9.13\times10^{-6}$; $\chi^2$ Bonferroni $p=4.77\times10^{-3}$; OR 0.47; 95% CI 0.33 to 0.66) (see online supplementary table S7). Among the five variants included in the *CBS* haplotype, the rs2124459 (c.1552+1199A>G) was the most significantly associated with a reduced risk of OFC in the 'mothers' group and reached an array-wide significance ($p=2.44\times10^{-4}$; Bonferroni $p=3.00\times10^{-2}$; OR 0.54; 95% CI 0.39 to 0.74, minor allele). In post hoc analysis, the *CBS* variant rs2124459 reached an array-wide significance for its association with a reduced risk of CP ($p=6.78\times10^{-5}$; Bonferroni $p=7.80\times10^{-3}$; OR 0.40; 95% CI 0.25 to 0.63, minor allele) but not with CL or CLP risk (table 1 and figure 1). Table 2 lists the nine top genetic variants that alongside the *CBS* rs2124459 were associated with OFC risk (three variants belonged to Hcy transsulfuration pathway (*CBS*), three to methionine cycle (*PCMT1*, *COMT*, *SHMT1*) and three to vitamin $B_{12}$ metabolism (*TCN2* and *CUBN*)); however, only rs2124459 reached array-wide significance.

**Table 2** Top significant variants associated with orofacial cleft risk in the discovery study on the 'mothers group'

| Marker | Chromosome | Position* | Gene name | Fisher's exact p value | Fisher's exact Bonferroni p value | OR (minor allele) | 95% CI | MAF, cases | MAF, controls |
|---|---|---|---|---|---|---|---|---|---|
| rs2124459 | 21 | 44475714 | *CBS* | $1.77\times10^{-4}$ | 0.02 | 0.53 | 0.38 to 0.74 | 0.37 | 0.52 |
| rs234709 | 21 | 44486964 | *CBS* | $7.66\times10^{-3}$ | 1 | 1.59 | 1.14 to 2.21 | 0.46 | 0.35 |
| rs4816 | 6 | 150114745 | *PCMT1* | $1.21\times10^{-2}$ | 1 | 0.65 | 0.47 to 0.91 | 0.34 | 0.44 |
| rs4920037 | 21 | 44481891 | *CBS* | $1.46\times10^{-2}$ | 1 | 1.64 | 1.10 to 2.45 | 0.26 | 0.18 |
| rs234705 | 21 | 44483772 | *CBS* | $1.83\times10^{-2}$ | 1 | 1.54 | 1.08 to 2.18 | 0.35 | 0.26 |
| rs737865 | 22 | 19930121 | *COMT* | $1.86\times10^{-2}$ | 1 | 0.66 | 0.47 to 0.93 | 0.29 | 0.38 |
| rs4820889 | 22 | 31019044 | *TCN2* | $3.26\times10^{-2}$ | 1 | 0.39 | 0.17 to 0.91 | 0.02 | 0.06 |
| rs2168781 | 17 | 18240746 | *SHMT1* | $3.71\times10^{-2}$ | 1 | 1.43 | 1.02 to 1.99 | 0.42 | 0.34 |
| rs1801229 | 10 | 17024615 | *CUBN* | $3.99\times10^{-2}$ | 1 | 0.56 | 0.33 to 0.95 | 0.08 | 0.13 |
| rs1801231 | 10 | 17024503 | *CUBN* | $4.13\times10^{-2}$ | 1 | 0.57 | 0.34 to 0.96 | 0.08 | 0.14 |

*Chromosome position according to GRCh37.
MAF, minor allele frequency.

### Children group

We tested the top significant haplotype block on *CBS* for its association with OFC risk in the French children group. The above-mentioned *CBS* haplotype '*Ddddd*' was significantly associated with a reduced risk of OFC ($\chi^2$ p=1.16×10$^{-2}$; OR 0.66; 95% CI 0.48 to 0.91). Consistently, the *CBS* variant rs2124459 was associated with a reduced risk of OFC (p=0.02; OR 0.70; 95% CI 0.52 to 0.95, minor allele). In post hoc analysis, rs2124459 was also associated with a reduced risk of CP (p=0.02; OR 0.61; 95% CI 0.40 to 0.93, minor allele) and CLP (p=0.03; OR 0.67; 95% CI 0.47 to 0.96, minor allele) but not CL (table 1 and figure 1).

### Replication study in the Belgian 'children' group

Since there was a significant association between the *CBS* rs2124459 and CP subtype in both French 'mother' and 'children' groups, we decided to primarily investigate the association between rs2124459 and CP in children from the Brussel's group. As expected, the rs2124459 was significantly associated with a reduced risk of CP (p=0.02; OR 0.64; 95% CI 0.44 to 0.93, minor allele). In contrast, there was no significant association with CL and CLP risk (table 1 and figure 1).

### LD analysis of *CBS* variants

Since the deleterious effect of the *CBS* variant rs5742905 (p. Ile278Thr) is antagonised by the insertion c.844_845ins68 through a skipping mechanism when in *cis* position,[22] we have specifically assessed through RFLP the occurrence of these two *CBS* variants and studied their LD with rs2124459 in a subset of 262 mothers and 237 children from the derivation study. LD patterns between the three variants were similar in the two subgroups. The two *CBS* variants c.844_845ins68 and rs5742905 were in high LD in both mothers (D′=0.95) and children (D′=1.00) (see online supplementary table S8). Online supplementary table S9 reports the LD pairwise analysis matrix output on the five *CBS* variants retained in haplotype analysis in the discovery study data set on 'mothers group'. The D′ values of rs2124459 were 0.96, 0.97, 0.83 and 0.83 for rs6586282, rs4920037, rs234705 and rs234709, respectively.

### Associations between the *CBS* rs2124459 variant and OCM biomarkers

The influence of the *CBS* variant rs2124459 on blood concentrations of OCM biomarkers was assessed in cases and controls from the mother's group and only in cases from the French children group. There was no significant association between rs2124459 and blood OCM biomarkers concentration. In exploratory analysis, in the subgroup of mothers' controls with low vitamin $B_{12}$ status (<25th percentile, n=23), rs2124459 was weakly associated with Hcy (p=0.04, Kruskal-Wallis test; p=00.1, trend test).

## DISCUSSION

Among 519 haplotypes tested for their association with OFC risk in the FePA study, the *CBS* locus reached array-wide significance. In genetic association analyses on per-variant basis, 10 top genetic variants, including the only variant having array-wide significance rs2124459, were found and belonged to the transsulfuration pathway of the OCM, the methionine cycle and the vitamin $B_{12}$ absorption and transport. We reported that the *CBS* rs2124459 was the single genetic predictor of OFC risk in both mothers and children. It was noticeable that this variant predicted a reduced OFC risk and was in strong LD with the other *CBS* variants associated with increased OFC risk in previous studies. Its risk prediction has been replicated in a second cohort recruited in Belgium. Furthermore, the minor allele of *CBS* rs2124459 was associated with a reduced risk of CP in both mothers and children groups. The association between *CBS* rs2124459 and a reduced risk of CL and/or CLP deserve to be replicated in independent studies.

CBS is a major enzyme of the Hcy transsulfuration pathway of OCM that needs vitamin B6 coenzyme pyridoxine as a cofactor for the conversion of Hcy to cysteine. CBS is preferentially expressed in liver and kidney in adults and in heart and neural tube in early stage of embryogenesis.[27] The *CBS* rs2124459 predicted a reduced risk of OFC in children and mothers, with a more pronounced influence in mothers. This variant has never been evaluated in former studies. In an Italian study, the *CBS* c.844_845ins68 was associated with an increased OFC risk in children when the insertion was inherited from the mother, in comparison to the fathers, suggesting a maternal effect.[28] Minor allele of intronic *CBS* variants rs4920037 and rs234705 were associated with an increased risk of OFC in Italia and Norway, respectively.[21 29] In a Norwegian population-based study, the minor allele of the synonymous polymorphism *CBS* rs234706 (c.699C>T) had protective effect on the 'CL with or without CP' phenotype only when found in mothers but not in children.[30] The FePA study did not confirm any of the previously reported associations between *CBS* c.844_845ins68, rs4920037 and rs234705 and the occurrence of OFC. This apparent discrepancy can potentially be explained in three points: (1) our study considered the role of the rs2124459 variant, which was not the case in other studies; (2) in our study, rs2124459 was in high LD with rs4920037 (D′=0.97) and rs234705 (D′=0.83), suggesting its potential role as a 'tag' variant for the *CBS* locus; and (3) in our study, rs4920037 and rs234705 had significant nominal p values before Bonferroni correction (rs4920037, p=1.46×10$^{-2}$; rs234705, p=1.83×10$^{-2}$), suggesting their potential significant association with OFC risk. This balancing effect between rs2124459 and other *CBS* variants needs to be assessed in independent studies.

There is limited evidence on the influence of *CBS* variants associated with OFC risk on Hcy plasma level. Data from the Women's Genome Health Study using a genomewide association study approach revealed a significant association between the *CBS* variants rs6586282 (c.1359–134G>A) and plasma Hcy level, assuming an additive model (β=−0.030).[31] This study did not show a significant association between plasma Hcy level and *CBS* variants related to OFC risk in the FePA study. Consistently, data from the European Atherosclerosis Research Study (Ears II Group) failed to demonstrate a significant association between *CBS* variants (c.844_845ins68, rs234706, rs1801181) and plasma Hcy level.[32] In the FePA study, *CBS* rs2124459 was associated with decreased plasma Hcy level in mothers' controls showing a low vitamin $B_{12}$ status. These data converge towards the need for further evaluation of the association between *CBS* variants and OFC risk through Hcy level modulation. Future population studies should assess the role of nutritional factors including metabolic and nutritional markers of vitamin B6 status on OFC risk.

In contrast to targeted studies of OCM genetic predictors of OFC risk, we did not found any association with *MTHFR* variants and in particular with rs1801133. This *MTHFR* polymorphism has been studied in several populations worldwide. Its influence on OCM and on neural tube defect risk depends strongly on environmental factors and in particular on the folate status of the studied population.[33 34] Our study was consistent

with these data, in regard to the normal folate status of the FePA population and the lack of influence of *MTHFR* variant on Hcy (data not shown). A study that has assessed 89 SNPs on 14 folate metabolism-related genes showed a risk association between *NOS3* and *TYMS* variants and non-syndromic CL with or without CP in non-Hispanic white subjects and an association with *MTR*, *BHMT2*, *MTHFS* and *SLC19A1* variants in Hispanic subjects.[35] Our results are also consistent with a previous case–control study of the French population, which found a beneficial effect of folate intake of mothers on offspring's risk and the lack of risk association of rs1801133 genotype.[36]

Among the 10 top variants associated with OFC risk in unadjusted analysis of the FePA study, 3 variants belonged to genes involved in the absorption and transport vitamin $B_{12}$, *TCN2* (rs4820889, rs1801229) and *CUBN* (rs1801231). We found an OFC risk prediction of the non-synonymous missense variant of *TCN2* rs4820889 (c.1196G>A; p.Arg399Gln) located in 22q11 in the unadjusted analysis on the 'mothers group' discovery study. It was in strong LD with *TCN2* rs1801198 ($D'=0.99$), a result which is consistent with the high LD in transmission of rs1801198 (c.776G>C; p.Arg259Pro) in cases–parents trios, in an Italian population.[37] The rs1801198 variant is known to influence the transcription of *TCN2* and the blood concentration of transcobalamin and Hcy.[38 39] *CUBN* is involved in the intrinsic factor-mediated absorption of vitamin $B_{12}$. A study on 391 CL/P triads from Italy assessed three genetic variants on *CUBN* and revealed no association with CL/P onset.[40] Among the top variants involved in the methionine cycle, *PCMT1* rs4816 (c.532G>A; p.Val178Ile) has been identified as a potential predictor of neural tube defects.[41]

We need to acknowledge limitations. First, the GoldenGate Assay applies stringent requirements to the quality of genetic variants and may generate missing data. It is the reason why we applied stringent quality control procedure by removing variants with call rates under 95%. Second, we applied a conservative method for multiple testing correction using Bonferroni procedure. However, one issue with Bonferroni correction is that it can lead to type II errors. Indeed, type I errors cannot decrease without inflating type II errors with the potential consequence of failing to detect variants with moderate or low effect size.[42] For these reasons, we have reported in table 2 the 10 top variants significantly associated with OFC risk according to Fisher's exact p value, highlighting other potential variants of interest.

In conclusion, the *CBS* rs2124459 was the single variant that reached array-wide significance among 10 top variants associated with OFC risk, including variants related to the Hcy transsulfuration pathway, methionine cycle and vitamin $B_{12}$ metabolism. The *CBS* rs2124459 predicted a reduced risk of OFC in French and Belgian populations, a result that is consistent with its LD with other variants previously reported as predictors of increased risk. These results highlight the prominent involvement of the vitamin B6-dependent transsulfuration pathway of Hcy in OFC risk and the need for evaluating the nutritional and metabolic markers of vitamin B6 status in further population studies.

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