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# Investigation of SLC30A4 and SLC39A14 Zinc Transporter Gene's Mutations in the Pathogenesis of Neural Tube Defects in Setif, Algeria

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### Authors' contributions

*This work was carried out in collaboration between all authors. Author RB designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors DT and NA managed the analyses of the study. Author RB managed the literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** This study was designed to investigate a common polymorphism in the exon 5 of the solute carrier SLC30A4 (ZNT4) gene 915 T-C in a group of mothers with neural tube defects (NTDs) babies compared to healthy controls in Setif region of Algeria, as well as the detection of a pathogenic mutation of the SLC39A14 (ZIP 14) gene in the NTD group.

**Methodology:** The case-control study, included 94 healthy mothers and 88 mothers with previous NTDs child; aged between 24 and 48 years. Peripheral blood DNA extraction was done by phenol-chloroform method. T915C polymorphism in ZnT4 gene was analyzed by polymerase chain reaction. Furthermore, sequencing of promoter 1: 333 base pairs of ZIP 14 gene was investigated. Odds ratio and Confidence Interval were calculated.

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**Results:** Our results revealed that homozygous mutant (CC) carriers in the control group were 6%, and in the NTDs mothers it was 7%, with a risk of 0.97 (CI 95%: (0.29- 3.26). The difference between the allelic frequency of the allele C among NTD s mothers compared to control mothers was not significant (Odds ratio 0.9, CI: 0.57 - 1.43). Sequencing of ZIP 14 gene didn't show any mutation and alteration in mothers with a previous NTD child.

**Conclusion:** The majority of pregnancies carrying neural tube abnormalities occur in Algerian mothers without previous NTDs cases. Furthermore, despite the lack of a relationship between zinc transporter genes and NTDs in our study, further investigations focusing on the molecular mechanisms and relevance of nutritional zinc status in relation with these malformations should be considered, attempting to find some highlights about pathogenesis of these defects in our country.

*Keywords: Neural tube defects; mutation; zinc transporter genes; ZIP14; ZnT4; Setif; Algeria.*

## 1. INTRODUCTION

Failure of neural tube closure by the 28th day post-conception leads to serious congenital malformations, such as anencephaly and spina bifida, more commonly known as neural tube defects (NTDs) [1]. They represent the second most common birth defect in the world, affecting 0.5-2 live births per 1000, with varying prevalence in different populations [2]. The etiology of NTDs is complicated, multifactorial and involves both genetic and environmental factors. Although folic acid supplementation is widely used by pregnant women to reduce the risk of NTDs [3], they still are important congenital malformations having wide implications. This may be due to the fact that deficiency of folic acid alone is not responsible for all kind of NTDs and many other factors (nutritional and genetic) are responsible in the etiology of various kinds of NTDs [4]. Moreover, a lack or an excess of trace elements and the interactions between vitamins and trace elements may play a significant role in their development [5].

Zinc (Zn), is an indispensable trace mineral, required for the structures and functions of many proteins, nucleic acids, carbohydrates, and lipids, playing a critical in biological activities, such as fetal growth and development, differentiation, survival, neural tube closure, cellular metabolism, neuromodulator in synaptic transmission and gene expression [6]. In addition, this element is also involved as cofactor for the enzymes in the metabolism of folate [6]. Zn homeostasis is tightly controlled by the coordinated activity of Zn transporters and metallothioneins, which regulate the distribution, storage, and intracellular and extracellular concentration of Zn. These transporters are divided into two major families, SLC39s/ZIPs and SLC30s/ZnTs, which transport

Zn in opposite directions through cellular and intracellular membranes [7]. The 14 members of the ZIP family have 8 putative transmembrane domains and are the first gateways for Zn uptake into the cells; these gateways elevate the intracellular cytoplasmic Zn content by an influx of Zn from extracellular fluid or intracellular organelles [8]. Whereas, the members of the SLC30 solute carrier subfamily share the same predicted structure, with six membrane-spanning domains and a histidine-rich intracellular loop between helices IV and V, excepted for ZnT-6 which retains a serine-rich loop [9]. The SLC39A14 gene (ZIP14, OMIM #608736) is located on 8q21.3 and has 13 exons [10]. Various experiments have demonstrated that ZIP14 transporter plays a major role in the mechanism responsible for hypozincemia that accompanies the acute phase response for inflammation and infection [11].

In contrast to the ZIP family, ZnT-family members reduce the intracellular cytoplasmic Zn content by effluxing Zn from the cytosol or transporting it into intracellular organelles or vesicles [7]. Higher levels of ZnT-4 are found in brain, mammary glands and epithelial cells [12]. The SLC30 A 4 gene (ZnT4, OMIM #AF025409) is located on 15 q21.1 and has 8 exons [13].

Numerous studies have shown that low maternal zinc status during pregnancy is linked to adverse pregnancy outcomes including abortion, fetal growth restriction, and NTDs [14]. In addition, either nutritional factors and/or genetic defects related to zinc may cause zinc deficiency among women which then, may predispose to NTDs babies [15,16,17,18,19], and increase prevalence of these malformations as observed concerning the polymorphism at exon 5 of the ZnT4 gene 915 T-C which may play a role in neural tube defects causing a 2.6 risk [20].

The fact that the biological mechanisms leading to the NTDs are still misunderstood in Algeria, it is likely that an alteration in the function of a gene unrelated to the ways of folic acid might play a role in these defects. The main purpose of this study was to investigate a common polymorphism in the exon 5 of the SLC30A4 (ZNT4) gene 915 T-C in a group of mothers with neural tube defects (NTDs) babies compared to healthy controls in Setif region of Algeria, as well as the detection of a pathogenic mutation of the SLC39A14 (ZIP 14) gene in the NTD group.

## 2. SUBJECTS AND METHODS

### 2.1 Study Population

This study was performed on a group of 94 apparently healthy women without any familial neural tube defects history, aged between 24 and 48 years (control group) and 88 age – matched mothers with a previous NTD child, from Setif maternity Hospital, Algeria. An informed consent for genetic analysis was obtained from participant.

### 2.2 DNA Sampling

Peripheral blood samples were collected, in EDTA tubes and frozen at -20°C until their transfer to Ankara/Turkey for DNA extraction by conventional phenol-chloroform method. DNA concentration and purity were quantified for each sample by spectrophotometry (Nanodrop ND-100).

### 2.3 Genotyping Analysis and Sequencing

Genotyping analysis was made by polymerase chain reaction (PCR) amplification in a thermal cycler (Biometra), using specific primers for SLC30A4 (ZNT4) gene exon 5: 915T-C, Forward: 5'-AGCAAGAAGGGACATATTCC-3' (Fermentas); and Reverse: 5'-GGTAAAAGATGGGAGAGTTC-3' (Fermentas), using 5 µl of 10 x PCR Buffer, 25 mM MgCl<sub>2</sub>, 10 mM of dNTP's mix, and 5 U/ µl. of Taq polymerase (Fermentas) in a total reaction volume of 50 µl. PCR conditions were as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min by 34 cycles. The products were separated on 3% agarose gel and visualized with ethidium bromide. Samples were genotyped in duplicate. The 3 genotypes were evaluated by restriction

with *Moraxella bovis* (Mbo I) (Fermentas) and shown on agarose gel [20].

In addition, following DNA extraction, promotor region 1 of the ZIP 14 (SLC39A14) gene was amplified by PCR reaction carried out in a reaction volume of 50 µl containing 100 ng of genomic DNA, 20 pmoles of each primer, 0.2 U/ µl. Taq polymerase (Fermentas), 200 µM of each d NTP and 2.5 mM MgCl<sub>2</sub>. The PCR reaction started after 5 min at 95°C, followed by 34 cycles of 50 s of denaturation at 94°C, 50 s of annealing at 52°C and 1 min extension at 72°C. Two different primer sets (Forward: 5'-TCACCCCAAATTAACATTTCT-3') and; Reverse: 5'-GCTAGCGGGTGAGAGCTTC-3') were used for amplifying the promotor region using a Biorad DNA Engine [21]. PCR revealed a 333 base pairs -amplified product. Two purification solutions (purification system KIT METIS) were added to the PCR product tubes and leaved for 2 h at 4°C. After centrifugation at 13000 rpm (4°C), the products were separated on 3% agarose gel and visualized with bromophenol blue. The samples were sequenced, using a DNA sequencer (Beckman Coulter CEQ 8000 Genetic Analysis System). The detailed experimental processes were performed according to the manufacturer's instructions.

### 2.4 Statistical Analysis

Genotype and allele frequencies of cases and control subjects were determined and the odds ratios (OR) as well as their 95% confidence intervals (CI) were calculated to evaluate the possible association between different genotypes and NTDs. A *P*-value less than 0.05 were considered as significant.

## 3. RESULTS AND DISCUSSION

The analysis of PCR products of the gene ZnT4 exon5 915 (T - C) after using the restriction enzyme (Mbo I) demonstrated the existence of 3 genotypes: wild type genotype (TT), heterozygote (TC) and homozygous mutant (CC).

Data on the distribution of the polymorphism 915 T - C of the ZnT4 gene is given in table 1. Our results revealed that CC carriers in the control group were 6%, and in the NTDs mothers it was 7%, with a risk of 0.97 (CI 95%: (0.29- 3.26). On the other hand, the heterozygous genotype (CT) has been identified in 43 mothers in the control

group (45.75%) and 36 (40.90%) in NTDs mothers group respectively. The difference between the allelic frequency of the allele C among NTDs mothers compared to control mothers was not significant (Odds ratio 0.9, CI: 0.57 - 1.43).

Regarding the ZIP14 gene, our data revealed no gene alteration related to neural tube defects in Algerian NTD mothers (Fig. 1).

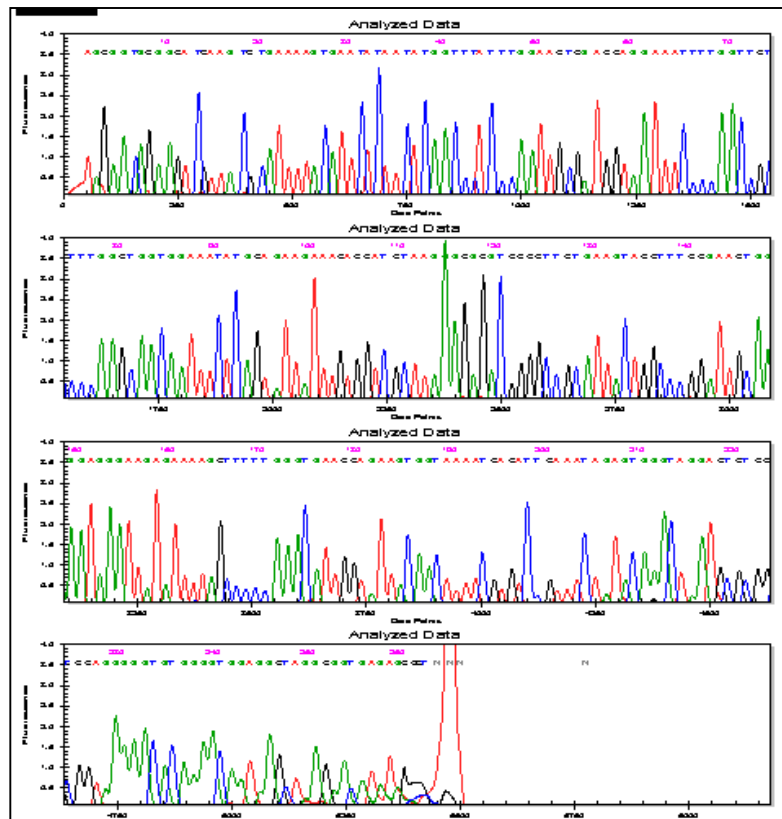
Zinc is a critical nutrient for a wide range of cellular machineries and for the development of central nervous system. A disturbance in Zn

homeostasis due to maternal zinc deficiency is a serious nutritional problem, and has pathogenic consequences [22], including children's retarded growth and development, spontaneous abortion, but the main teratogenic effect of such state during pregnancy seems to be a defective closing of neural tube [23]. It was estimated that 82% of pregnant women worldwide usually have an inadequate regular intake of zinc and suffer health consequences of zinc deficiency [24]. Moreover, Zn plays a role in the absorption of folate in the gastrointestinal tract, therefore zinc deficiency can cause malabsorption of food folate [25].

**Table 1. Genotype and allele frequency of the ZnT4 915 T - C polymorphism**

Genotype	Control mothers n (%)	NTDs mothers n (%)	Odds ratio (95% CI)
TT	45 (0.48)	46 (0.53)	1
CT	43 (0.45)	36 (0.40)	0.81 (0.44-1.49)
CC	6 (0.06)	6 (0.06)	0.97 (0.29- 3.26)
Allele T	133 (0.7)	128 (0.73)	1
Allele C	55 (0.29)	48 (0.27)	0.90 (0.57 -1.43)

\* Odds ratio (95% CI) vs. controls.



**Fig. 1. Promotor 1 region sequence of ZIP 14 gene in NTD mothers**

Vegetarians, and the malnourished are at increased risk for zinc deficiency because the bioavailability of zinc in vegetarian diet with abundant phylate is low, and this impaired Zn absorption and increased excretion of zinc [26].

NTD's are congenital multifactorial disorders arising from a complex combination of genetic and environmental interactions involving nutritional deficiencies, genetic predisposition, in addition to some trace elements and vitamins that could partially explain these anomalies [27]. Zinc deficiency is one of the possible factor for the etiopathogenesis of NTD's [28].

Previous data in human studies have shown a possible role of zinc metabolism in at least some of the NTDs mothers [18,19,27,29]; who have defective zinc absorption due to chronic zinc deficiency, which returned to normal after zinc supplementation [15].

Zinc supplementation can reduce the risk for certain pregnancy complications, including congenital defects, by preventing primary deficiencies caused by diet or by treating secondary deficiencies [30].

To our knowledge, this is the first study conducted to evaluate the possible association of zinc transporter genes with neural tube defects in Setif, Algeria.

Recently, Yan et al. [31] found that lower concentrations of Zn and other essential trace metals in maternal hair samples during the early period of pregnancy were associated with an elevated risk of NTDs in offspring. In addition, in her study, Abdulhussain [32] found that there was an association between low maternal serum zinc level and conception with fetuses with NTDs, and that these levels decreased when gestational age increased and this can be explained by the physiological changes that occur in pregnancy.

Mutations in ZIP transporters are currently known to be associated with genetic diseases in humans [33]. Mutations in SLC39A4, encoding ZIP4, cause acrodermatitis enteropathica (AE), an autosomal recessive disorder affecting the uptake of zinc, disrupting intestinal zinc absorption and causing systemic zinc deficiency that can be reversed by effective oral zinc supplementation [34]. In addition, there was one spontaneous abortion and two major NTDs in patients with AE, but the pregnancy outcome

was good when the patient was given supplemental zinc throughout her pregnancy [35].

The involvement of ZnT4 (915 T - C) polymorphism in the pathogenesis of NTDs in setif, Algeria could not be confirmed in the present study, despite the fact that this polymorphism seemed to be a good marker for spina bifida and in particular in cases where low zinc concentrations were observed according to the study which focused on 105 mothers of children with NTDs in Turkey, and where homozygosity (CC) on gene ZnT4 was higher in NTD mothers than control group; bringing a twofold risk [20].

In agreement with the study of Torun et al. [21], our results don't reveal any relationship between neural tube defects and ZIP 14 gene in Algeria.

It has been recently reported that the homozygous loss-of-function mutations of ZIP14 cause progressive parkinsonism-dystonia and neurodegeneration with hypermanganesemia in childhood [36].

A crucial role of Zinc transporter ZIP12 for neurulation was reported linking dietary zinc deficiency in humans to neural tube defects [30]. The study of Chowanadisai et al. [37] showed that the ZIP12 is critical for neuronal differentiation, neurulation, and embryonic development. In addition, the defects in neuronal maturation and neural tube closure caused by ZIP12 inhibition are consistent with the localization and expression of mZIP12 and xZIP12. Zinc transport by ZIP12 may support an increased demand for this element during early nervous system development. Furthermore, during embryogenesis, knockdown of ZIP12 expression impaired neural tube closure and arrested development.

#### 4. CONCLUSION

The birth of an NTD child is a personal tragedy for him and for his family especially in undeveloped countries. In our study we analyzed involvement of two zinc transporter genes suspected in the etiology of neural tube defects in a group of NTDs mothers compared to healthy controls in an Algerian population. We studied the possible association of Znt4 (915 T - C) polymorphism with NTDs, and /or alteration in the promotor 1 region (333 base pairs ) of the Slc39a14(ZIP 14) .Although we were not able to

show a link between neural tube defects and these zinc transporter genes, further investigations focusing on the molecular mechanisms and relevance of other zinc transporter genes as well as the nutritional zinc status in pathogenesis of these malformations should be considered, attempting to find some highlights about pathogenesis of these defects in our country.

### ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

### ACKNOWLEDGEMENTS

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### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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