Role of nutrition, lifestyle factors, and genes in the pathogenesis of congenital diaphragmatic hernia: human and animal studies

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Congenital diaphragmatic hernia (CDH) is a severe malformation with a largely unknown pathogenesis. Because an unequivocal genetic relation is diagnosed only in a minority of patients, the involvement of multiple genetic and environmental factors is suggested. Although periconceptional environmental exposures, such as maternal malnutrition and unhealthy lifestyle factors, are associated with several birth defects, they have scarcely been investigated in CDH. Nutrition and lifestyle factors can be modified and may, therefore, contribute to the prevention of CDH. This review provides an overview of the human studies in which the influences of nutrition and some related lifestyle factors during embryogenesis of the diaphragm are described. In addition, the findings in humans are further substantiated by animal studies and the nutrient-gene interactions involved are elaborated upon. The information presented here contributes to the elucidation of the pathogenesis of CDH and will assist the development of preventive nutritional strategies in the future.

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INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a malformation characterized by a defect in the diaphragm. Worldwide, every year approximately 120,000 children are born with CDH.1-3 The intrauterine and perinatal mortality rate of CDH is around 60%, though it varies between 20% and 70%.4,5 The apparent increase in postnatal survival is probably biased by an increasing number of pregnancy terminations in patients with a poor prognosis.6 The birth prevalence rate of CDH is comparable among countries worldwide, but ethnic differences are reported.1,7,8 In white children, the frequency of CDH is higher than that in children of Asian, African American, or Hispanic ethnicity.9-12

Maternal drug use has been associated with the occurrence of CDH in the child,13-19 including antiepileptic drugs,20 antifungal drugs,21 and selective serotonin reuptake inhibitors.22,23 Most reports, however, are sporadic and most data are from epidemiological studies on congenital anomalies that include CDH but consider it as part of one heterogeneous group. No studies have been performed specifically on the proportion of CDH that is associated with the maternal use of medication during the period in which CDH develops.

In 30% of cases, CDH is associated with another malformation.5 Within this group, 50% of cases are due to a syndrome or genetic defect. This means that the cause of CDH is unknown in 85% of all cases. The morbidity of CDH is high, with pulmonary and gastrointestinal...
sequelae being most frequent.\textsuperscript{24} The most important challenge in the immediate newborn period is the “control” of pulmonary hypertension before surgical closure of the diaphragm defect.\textsuperscript{4} Because of the severe burden for patients and their families, a long-term follow-up program by a multidisciplinary team is often provided.\textsuperscript{25}

In this review, the embryologic background of CDH is described and related to the role of developmental and candidate genes in the interaction with nutrients and some related lifestyle factors in humans that are further substantiated by animal studies. The elaboration on nutrient-gene interactions will improve our understanding of the underlying mechanisms in the pathogenesis of CDH. This will potentially lead to nutritional interventions for CDH prevention in the future.

**EMBRYOGENESIS OF THE DIAPHRAGM**

In the classical view, the diaphragm is formed by the fusion of four different embryonic structures: 1) the septum transversum (the primordial diaphragm), 2) the pleuroperitoneal membrane, 3) the esophageal mesenchyme, and 4) the paraxial mesoderm of the body wall (Figure 1).\textsuperscript{26,27} Muscle precursor cells migrate and differentiate within the septum transversum and eventually form the diaphragmatic muscle. These processes take place between embryonic weeks 4 and 10. CDH occurs if the diaphragm fails to close in this embryonic period. As a consequence of the defect, the abdominal content “invades” the thoracic cavity and limits pulmonary growth. It is believed that the lungs are already hypoplastic, independent of the mechanical obstruction and limitation in fetal breathing movements.\textsuperscript{28} The defects observed in CDH are in general classified into posterolateral (Bochdalek), non-posterolateral or anterolateral (e.g., Morgagni), and central (Pentalogy of Cantrell), but they are not always limited to these regions. An eventration is an abnormally thin diaphragm caused by a muscularization defect and the word describes the type of the defect rather than the location. Sometimes a thin fibrous sheet covers the intestinal organs inside the thorax. This “sac” is different from an eventration and is not considered a separate type of CDH. It illustrates the importance of the precise phenotyping of CDH and the potential to correlate the defects with those reported in genetic models. This may lead to a better understanding of the deranged processes and pathways in CDH.\textsuperscript{29}

To date, our knowledge of cell biological processes and regulation of the temporospatial expression of genes implicated in human diaphragm development is scarce. Evidence suggests that a defect in the pleuroperitoneal folds (PPFs) eventually leads to defective diaphragm formation in mice.\textsuperscript{30–34} If this is proven true, the classical theory of the embryogenesis of the diaphragm must be revised.

**NUTRITION DURING EMBRYOGENESIS OF THE DIAPHRAGM**

From gametogenesis throughout pregnancy, the conceptus is compelled to adapt at the transcriptional level to changes in its environment determined by the maternal nutritional status, metabolism, and lifestyle. The maternal diet contains essential components that serve as building blocks, transcription factors, and intermediates for cell signaling in the embryo and fetus. With increasing knowledge of the role of nutritional factors in developmental pathways, evidence is increasing that an optimization of maternal nutrition can contribute to a reduction in the risk of congenital malformations.\textsuperscript{35,36}

From the moment of conception, the transfer of nutrients is by simple passive diffusion via the oviduct, yolk sac, extra-embryonic coelom, amniotic membranes, amniotic cavity, and intervillous space.\textsuperscript{37} The development of the placenta starts at the moment of implantation, i.e., the first week after conception. However, it is not earlier than after dissolution of the trophoblastic plugs at the end of the first trimester that maternal blood progressively flows within the intervillous space and nutrition is provided hemotrophically.\textsuperscript{37,38} Besides transporting nutrients, the placenta also actively synthesizes nutrients, hormones, and peptides that are needed for growth and differentiation of the fetus. With respect to CDH, it is important to realize that at the time of defective closure of the diaphragm, the embryo is dependent on nutrient and oxygen exchange via the amniotic membranes, which at that time of pregnancy also have some metabolic functions.\textsuperscript{39–41}

Deficiencies or an excess of nutrient supply to the embryo can be caused by maternal nutrient intake, disturbances in the absorption and transfer of nutrients to the embryo, increased nutritional needs, and genetic alterations in the mother and the child. It is likely that inadequate nutritional supplies within a certain time-frame affect the embryonic nutritional status and subsequent pathways. Derangements in these pathways may be implicated in CDH, and primary prevention of CDH by nutrition is feasible when the causes can be identified and treated, preferably in the preconception period.

**Vitamin A and CDH**

Vitamin A (retinol) is a fat-soluble vitamin that controls many developmental genes, mainly by its biologically active metabolite retinoic acid (RA). Humans are not able to synthesize retinoids de novo and therefore obtain this vitamin through the diet. During transport from the liver...
**Embryology**

embryonic day / weeks after conception

![Embryology diagram](image)

**Defects**

- **Posterolateral without rim (Bochdalek)**
  - Defect: RARa/RARb2 Wt1

- **Posterolateral with rim (Bochdalek)**
  - Muscularisation defect: SF/HGF

- **Central**
  - Rupture: Lox
  - Muscularisation defect: Gata4 Slit3

- **Eventration**

- **Anterior**
  - Muscularisation defect: Pax3 Cmet Fog2 Gab1 MyoD Myogenin

- **Morgagni**

![Defects diagram](image)

**Figure 1** Schematic overview of diaphragm development. The expression of genes in the components of the diaphragm are indicated by arrows. A–E: schematic overview of defects that are found in CDH and animal models. Underlined genes are those involved in the vitamin A pathway.
to other tissues, retinol is almost solely bound to retinol-binding protein (RBP). Cellular uptake of retinol is mediated by the recently discovered RBP receptor STRA6 and lecithin:retinol acyltransferase. Inside the cell, retinol is transformed to RA via a two-step oxidation. RA enters the nucleus and binds the nuclear receptors RAR (retinoic acid receptor) and RXR (retinoid X receptor), which heterodimerize to the promoter region of a gene (Figure 2).

The control of RA-dependent processes is achieved by the temporospatial expression of retinoid receptors and binding proteins as well as by time-dependent changes in vitamin A metabolism. Vitamin A metabolism is regulated by the interplay of three enzyme families: alcohol dehydrogenases, short-chain dehydrogenases, and members of the cytochrome P450 family.

From the first trimester onward, the amount of retinol supplied to the fetus is maintained at a constant level until maternal stores are almost completely depleted. Because the fetus produces its own RBP, only free retinol passes the placental barriers. The supply of retinoids to the embryo is not limited to retinol, because retinyl esters packed in lipoproteins and RA can also be taken up. Moreover, the fetal membranes have an active metabolic and transport function for retinoids.

The relationship between CDH and vitamin A has emerged from human studies and animal experiments. Yang et al. observed an increased risk of CDH in children born to mothers with low intakes of retinol. This is in line with the study of Finnell et al. suggesting that the elevated incidence of conotruncal heart defects in non-Hispanic whites might be related to lower vitamin A intake in this group. Because the same differential ethnic pattern has been observed for CDH, this association may also be of interest for further investigation. It is, however, remarkable that the prevalence of CDH at birth is not high in countries where vitamin A deficiency is endemic. This might be due to inadequate registration of congenital anomalies, including CDH.

Nutritional experiments in rats have shown that dams fed a vitamin-A-deficient diet give birth to offspring with multiple congenital anomalies, including abnormalities of the diaphragm, heart, limbs, vertebral column, ocular tissues, respiratory system, and cardiovascular system as well as segmentation defects. The most extensively studied animal model is based on nitrofen, an herbicide that disturbs the RA pathway. When nitrofen is administered to the pregnant dam, it results in CDH in a majority of the offspring, with the CDH phenotype being comparable to that observed in vitamin A deficiency.

Figure 2  Schematic overview of the vitamin A pathway. Depicted are the interactions with other pathways and structures that are discussed in this review. Abbreviations: TTR, transthyretin; Rol, retinol; RBP, retinol binding protein; STRA6, stimulated by retinoic acid 6; CRBP, cellular retinol binding protein; Ral, retinal; ADH, alcohol dehydrogenase; RALDH, retinaldehyde dehydrogenase; RAR, retinoic acid receptor; RXR, retinoid X receptor; RARE, retinoic acid response element.
deficiency models and in RAR knockouts.\textsuperscript{56–59} Nitrofen has been shown to block the enzyme retinal dehydrogenase 2 (RALDH2), resulting in RA deficiency (Figure 2).\textsuperscript{60} Supplementation of vitamin A or RA in the maternal diet counteracts the effect of nitrofen.\textsuperscript{61,62} The other antioxidants vitamin C and E also seem to have this protective effect, although only in lung and heart tissues.\textsuperscript{63–65} To date, no known reports describe the effects of nitrofen in humans or other animals except for certain rat strains with differences in nitrofen susceptibility.

Teratogenic effects of an excess of dietary vitamin A have been reported in humans and animals.\textsuperscript{66–69} The effect of high vitamin A exposure by vitamin-A-containing drugs in humans, e.g., the synthetic retinoid isotretinoin, has been described as the retinoic acid embryopathy.\textsuperscript{70,71} This syndrome includes craniofacial, cardiovascular, thymic, and central nervous system malformations and shows a remarkable similarity with those observed in hypervitaminosis A, but it lacks a diaphragm defect.\textsuperscript{71} To date, only one study, by Major et al.,\textsuperscript{72} showed higher levels of retinol in mothers of children with CDH. Because their children showed lower vitamin A and retinol-binding protein (RBP) levels in cord blood as compared to healthy control pregnancies, it is questionable whether a high or a low vitamin A exposure in early pregnancy is teratogenic. Although the results were significant, these results should be interpreted with care because the number of patients was very low. Moreover, it is not clear whether the postnatal vitamin A status of mother and child is representative of the status in the critical period early in pregnancy.

In conclusion, evidence from human studies, supported by animal models, suggests that a disturbance in vitamin A metabolism, resulting in vitamin A deficiency, might be involved in the development of CDH. Prospective studies and in vitro experiments are needed to obtain more insight into the underlying mechanisms.

Folate and CDH

Folate is an essential B vitamin present in green vegetables and meat. Folate plays an important role in several cell biological processes, such as amino acid metabolism, DNA synthesis, and methylation of DNA and RNA. The synthetic form, folic acid, is used in vitamin supplements and fortification of food because of its higher bioavailability and stability. Folate is a known modifier of the risk of birth defects in humans.\textsuperscript{73} Periconceptional use of a folic-acid-containing supplement reduces the occurrence of neural tube defects, congenital heart defects, and orofacial clefts.\textsuperscript{74–79} These birth defects have also been related to polymorphisms in genes that encode for proteins involved in folate metabolism, resulting in folate deficiency.\textsuperscript{76,80} A comparable preventive effect against CDH has been shown by high dietary B vitamin intakes.\textsuperscript{12,50,81} These findings are supported by studies in mice showing that maternal folate deficiency leads to an abnormal cell count and structure of the diaphragm.\textsuperscript{82}

One explanation of the protective effect of folic acid on birth defects is based on the reduction of high homocysteine levels. It is unknown, however, whether hyperhomocysteinemia is a primary teratogen or an epiphenomenon in the pathogenesis of these complex birth defects. It has been hypothesized that hyperhomocysteinemia causes excessive oxidative stress and may interfere with the folate receptor.\textsuperscript{83} Homocysteine also inhibits RA synthesis,\textsuperscript{84} and administration of RA to homocysteine-treated chicken embryos increased embryonic survival and diminished the number of congenital anomalies.\textsuperscript{84} So far, hyperhomocysteinemia has not been directly linked to CDH.

LIFESTYLE FACTORS AND CDH

Lifestyle factors, such as smoking and alcohol use, have been associated with several birth defects. Smoking is the strongest lifestyle factor associated with birth defects and is often accompanied by poor nutrition.\textsuperscript{85,86} Tobacco smoke contains numerous nicotine-containing compounds shown to disrupt vascular formation and retinoid homeostasis, thereby predisposing to birth defects.\textsuperscript{87} Although abnormal vascular formation and retinoid homeostasis might be pathogenic factors in CDH, a relationship with human CDH has not been identified. In contrast, smoking has also been associated with a lower risk of conotruncal heart defects and neural tube defects.\textsuperscript{88}

It is well known that excessive use of alcohol by pregnant women causes fetal alcohol syndrome, in which CDH has not been reported.\textsuperscript{89,90} In a recent paper, however, Felix et al.\textsuperscript{91} found a relationship between CDH and maternal social alcohol use in the months before conception and during the first trimester. In contrast to excessive alcohol use, social alcohol use is poorly defined but can be described as the regular use of low amounts of alcohol. There is some evidence that the metabolic pathways of alcohol and vitamin A interact. Alcohol may reduce RA status by inhibition of vitamin A metabolism and induction of RA degradation and thereby potentiate the teratogenicity of vitamin A deficiency.\textsuperscript{39} Furthermore, alcohol seems to aggravate the adverse effects of an excess of vitamin A, which has been demonstrated by the simultaneous administration of both ethanol and vitamin A in rats.\textsuperscript{92} In addition, excessive alcohol use is associated with poor nutritional intake, including low B vitamin intake. Whether this is critical to the preventive effect of peri-
conceptional folic acid and multivitamins against CDH should be further investigated. Obesity is a general feature of unhealthy lifestyles and is associated with malnutrition and reduced exercise. Prepregnancy obesity, defined as a body mass index higher than 30, is a risk factor for CDH. There is some evidence that the accompanying poor glycemic control and insulin resistance contribute to the pathogenesis of CDH. It seems that the teratogenicity of hyperglycemia is related to abnormal glycosylation of proteins, increase in oxidative stress, reduction of antioxidants, and derangement of gene expression. Of special interest is the reduction of the levels of Pax-3 in diabetes. Pax-3 has been related to neural tube closure but is also involved in development of the diaphragm (Table 1). The expression of Pax-3 can be improved by vitamin A, at least in cardiac cells in the nitrofen model.

**GENETIC FACTORS INVOLVED IN DIAPHRAGM DEVELOPMENT**

Although genetic defects and several candidate genes have been identified in patients with CDH, most knowledge on genes and CDH is derived from animal studies. The function of these genes can be classified into transcription factors and factors involved in cell signaling, in cell migration and mesodermal patterning, and in extracellular matrix biosynthesis (see Figure 1 and Table 1). The following paragraphs describe only those genes related to known nutritional or lifestyle factors. For an extensive overview of other genes that may be involved in CDH, we refer to several excellent reviews in the literature.

**Transcription factors**

The transcription factor COUP-TFI works together with GATA4 and FOG2 and is involved in RA (vitamin A) metabolism. All three genes are located on the frequently altered chromosome regions on chromosomes 15 (COUP-TFI) and 8 (GATA4 and FOG2) in human CDH. In mice, the modulation of these genes induces a muscularization defect. Mutations in Wilms’ tumor 1 (WT1) cause a spectrum of syndromes, including CDH. The WT1 gene resides in the region on chromosome 11 that is frequently altered in human CDH and is suggested to share the same mode of pathogenesis. Migratory muscle cell precursor cells in the septum transversum express the transcription factor Pax3, which is also expressed in neural crest cells that emerge from the dorsal neural tube. In the Splotch mouse, the knockout of Pax3 leads to an amuscular diaphragm. Double mutants of the nuclear RAR (RARα/RARβ2) develop CDH. This finding suggests a disturbance in the retinoid-signaling pathway contributes to the pathogenesis of CDH.

**Factors in cell signaling**

The human Matthew-Wood syndrome, which includes microphthalmia and CDH, is caused by a mutation in STRA6. Recently, STRA6 has been identified as the cellular receptor for retinol. STRA6 has an important regulatory function in cellular vitamin A homeostasis in cooperation with lecithin:retinol acyltransferase (LRAT).

**Cell migration and mesodermal patterning**

Glypican-3 (GPC3) is expressed selectively in mesodermal-derived tissues and functions as a regulator of growth factors, apoptosis, and guidance molecules, such as the Slit-Robo complex. A role for GPC3 in the regulation of insulin-like growth factor 2 has been proposed, and mutations cause Simpson-Golabi-Behmel syndrome (SGBS). Patients with SGBS have a defect in cholesterol synthase, which leads to a fetal-placental overgrowth syndrome with CDH in some cases. So far, interactions between genes involved in cell migration and mesodermal patterning and nutrition and lifestyle factors are not reported.

**Extracellular matrix**

The impairment of extracellular matrix formation leads to a variety of developmental defects. In mice, a deficiency of fibrillin-1 causes heart and lung anomalies that can be prevented by the medicine losartan, an angiotensin-II receptor antagonist, or a transforming growth factor (TGF)-β neutralizing antibody. Mutations in HCCS cause MIDAS syndrome, which is characterized by microphthalmia and CDH. This combination of features has also been observed in the aforementioned Matthew-Wood syndrome.

In summary, several genetic factors in several pathways are associated with CDH. So far, some evidence is available that interactions between genetic factors and the periconceptional status of maternal vitamin A, glucose, and cholesterol may play a role in the pathogenesis of CDH.

**CONCLUSION**

It is clear from human and animal studies that CDH has a heterogeneous phenotype. This suggests the involvement of multiple pathways, mechanisms, and interactions. Most evidence of a nutrient-gene interaction in...
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| COUP-TFII (NR2F2)              | Human   | Vitamin A pathway  | 15q26.1-26.2   | Posterior defect                              | You et al. (2005)\(^{106}\)  
Klaassens et al. (2005)\(^{98}\)  
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Ackerman et al. (2005)\(^{106}\) |
| Coup-TFII                      | Mouse   | Vitamin A pathway  | 8p23.1         | Posterior defect                              | Shimokawa et al. (2005)\(^{134}\)  
Slavotinek et al. (2006)\(^{133}\)  
Jay et al. (2007)\(^{107}\)  
Lopez et al. (2006)\(^{135}\)  
Temple et al. (1994)\(^{136}\)  
Howe et al. (1996)\(^{137}\)  
Ackerman et al. (2005)\(^{98}\)  
Ackerman et al. (2005)\(^{106}\) |
| GATA4                          | Human   | Vitamin A pathway  | Genetic defect | Central muscularization defect                |                     |
| Gata4                          | Mouse   | Vitamin A pathway  | Gata 4 +/-    | Central hernia (sac)                          |                     |
| FOG2 (ZFPM2)                   | Human   | Vitamin A pathway  | Mutation 8q22-23 | Muscularization defect                        |                     |
| Fog2 (Zfpm2)                   | Mouse   | Vitamin A pathway  | Knockout       | Muscularization defect of posterior diaphragm, 
     bilateral                                      |                     |
| WT1                            | Human   | Possible; same mechanism as nitrofen and vitamin A deficiency models | Heterozygous loss of function | Diaphragm defects WAGR, Denys-Drash, Meacham and Frasier syndromes |
| Wt1                            | Mouse   |                      | Knockout       | Posterior lateral hernia                       |                     |
| Pax3 (Splotch)                 | Mouse   | Maternal diabetes reduces Pax3 expression in embryo. Vitamin A stimulates Pax3 expression | Mutant | Amuscular diaphragm                            |                     |
| Rarγ/Rarβ2                     | Mouse   | Vitamin A pathway  | Double mutant  | Posterior diaphragmatic hernia                |                     |
| STRA6                          | Human   | Vitamin A pathway  |                | CDH or eventration                            | Pasutto et al. (2007)\(^{112}\)  
Kawaguchi et al. (2007)\(^{42}\) |
| Factors in cell migration and mesodermal patterning | GPC3 Glypican 3 | Cholesterol?? | Mutation | Diaphragmatic hernia, Simpson-Golabi-Behmel syndrome | Pilia et al. (1996)\(^{135}\)  
Hughes-Benzie et al. (1996)\(^{136}\)  
Veugelers et al. (2000)\(^{40}\)  
Li et al. (2001)\(^{141}\) |
relation to CDH is available from human and animal studies on vitamin A. The homeostasis of the vitamin A pathway is under strict control. Disturbances in this signaling pathway during pregnancy can be harmful to the embryo due to derangements in retinoid levels, binding proteins, and converting enzymes. Among species, there are different sensitivities to retinoids due to variations in placental transfer of RA isomers or species-specific transcription factors, resulting in different outcomes. Phenotypic differences have been observed in animal models of CDH. In humans, the differences in nutritional habits and other environmental factors may contribute to a different susceptibility and phenotypical outcome, possibly in more subtle ways than can be found by gene-expression analyses alone. Some nutritional and lifestyle factors that potentially disturb retinoid homeostasis are alcohol use and smoking. However, there is little evidence thus far, and the extrapolation of knowledge derived from animal models to the human situation is still limited.

Both vitamin A deficiency and excessive vitamin A intake are suggested to be teratogenic. Therefore, the vitamin A recommendation for pregnant women is 800 μg retinol activity equivalents (RAE) per day, with a limit of 3,000 μg RAE. A minimal recommendation is important because of the antioxidant capacity of vitamins A, C, and E and their demonstrated protective effect in humans and animal models of CDH. This effect may suggest that nutrient-gene interactions are implicated in CDH, but further support in the form of human studies is needed.

The co-occurrence of CDH and facial, thyroid, and cardiac anomalies, whether or not within a defined syndrome, suggests the existence of a dysmorphogenic mechanism in which the neural crest is involved. The neural crest is an important embryonic structure from which regulatory signals are transmitted and cells migrate to form the organs. The development of the neural crest is influenced by modifiers like retinoids and folic acid. Therefore, it would be very interesting to study nutrients and related genes further in relation to CDH and the neural crest.

One way to summarize the mechanisms involved in CDH is illustrated by the mesenchymal hit hypothesis. This hypothesis is an extension of the dual hit hypothesis formulated by Keijzer et al. which suggests that in CDH, the following occurs: 1) similar signaling pathways are involved in the differentiation of mesenchymal cells in all of the affected organs; 2) the function of these mesenchymal cells is disrupted by genetic or environmental triggers. The goal for future research is to identify and further delineate these pathways.

The investigation of the pathways involved and the interactions between nutrients, lifestyles, and genetic factors creates opportunities for optimizing the preconception maternal diet. This may ultimately lead to the prevention of CDH or at least to a decrease in the amount of hypoplasia or the size of the defect. The relatively low rate of CDH at birth and the subtle effects of nutrition and lifestyle factors emphasize the need for large epidemiologic studies in which international collaborations with access to birth registries are necessary, such as the recently instituted EURO-CDH consortium and the CDH registry.

Acknowledgments

Declaration of interest. The authors have no relevant interests to declare.

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