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**Abstract**

While breastfeeding is the recommended feeding mode in infancy, rates are low in some Western societies, and infants are widely fed formula. France, in particular, shows high rates of infant formula use, including formulas with protein hydrolysates. The degree of protein hydrolysis has previously been associated with neurodevelopmental outcomes. The present study examines the associations between the protein's hydrolysis degree in infant formula and child neurodevelopment up to 3.5 years of age in the French nationwide ELFE study. Parents reported on brand and name of the formula used at 2 months and protein hydrolysis degree was derived from the ingredient list. Analyses were based on 6979 infants (92.2%, 6.8% and 1% consuming non-hydrolysed, partially and extensively hydrolysed formulas, respectively). Neurodevelopment was assessed at age 1 and 3.5 years with the Child Development Inventory (CDI), at age 2 years with the MacArthur-Bates Communicative Development Inventories and at age 3.5 years with the Picture Similarities sub-scale (British Ability Scales). Associations between protein hydrolysis degree and child neurodevelopment were assessed using linear and logistic regression for overall scores and poor CDI sub-domain scores (<25$^{th}$ centile), respectively. Among formula-fed infants, protein hydrolysis degree in infant formula was not associated with overall neurodevelopmental scores up to 3.5 years. Some associations were found with the motor skills CDI sub-domain but they were not consistent at 1 and 3.5 years as well as across sensitivity analyses. The use of hydrolysed formula appears safe in terms of overall neurodevelopment, and research should further investigate specific neurodevelopmental domains.

**Keywords/phrases**: infant formula; protein hydrolysates; motor skills; language; cognition; neurodevelopmental scores

## Introduction

Exclusive breastfeeding is recommended for up to 6 months of age and supports the health of the mother and the child [1; 2]. In particular, there is a consensus about the beneficial effects of breastfeeding on child neurodevelopmental outcomes, which is supported by well-established associations [3; 4]. While an overwhelming 90% of infants still receive breastmilk at age 6 months globally, one out of two infants from high income countries do not receive any breast milk at this age, with some Western countries showing even lower rates [5]. This is evident in France, a country with traditionally low rates of breastfeeding, where the corresponding rate of formula-fed infants (not necessarily exclusively) is high [6] and parents use a broad range of infant formulas [7; 8]. Comprehensive nationwide data demonstrate that the use of formulas with varying degrees of protein hydrolysates ranges between 2% and 7% for extensively and partially hydrolysed forms, respectively [7]. According to European regulations it is required to compare hydrolysed formulas against an approved control formula (hydrolysed or non-hydrolysed) in order to establish adequate growth among infants [9]. However, there is scarce evidence of possible effects of hydrolysed formulas on neurodevelopmental outcomes among formula-fed infants [10].

Hydrolysed formulas include proteins that have been broken down partially or extensively in order to facilitate easier transition through the gut and decrease the likelihood of an immune reaction [11; 12]. However, the efficacy of partially hydrolysed formulas in primary prevention of allergies is still debated [8; 13; 14; 15]. According to the European regulatory framework, each new hydrolysed formula needs to be evaluated on an individual basis to ensure its safety and suitability, in addition to meeting the nutritional requirements of the infant [16]. Different randomised controlled trials (RCTs) have reported adequate physical growth among children who consumed hydrolysed formulas[17; 18; 19; 20; 21]. Moreover, some RCTs have shown growth among children consuming a non-hydrolysed formula to be accelerated compared to children consuming an extensively hydrolysed formula [17; 20; 21]. However, only one RCT has examined the influence of protein hydrolysed infant formula on neurodevelopmental outcomes. In this trial, infants fed with extensively hydrolysed formula up to 8.5 months of age had more favourable cognitive outcomes during the first year of life, compared to those fed with regular cow-milk formula [10].

Research that has distinguished between different types of formulas according to their content in protein hydrolysates has oftentimes drawn links between hydrolysed formulas and breastmilk ─the gold standard for infant feeding─, insofar as they both contain free amino

acids. Human milk is characterised by a high content in free amino acids (in particular glutamate), which is seven times higher in extensively hydrolysed formulas [22; 23]. However, free amino acids are rare in non-hydrolysed formula [24]. As outlined above, there is preliminary evidence from an RCT involving term infants, which has implicated extensively hydrolysed formulas (with a high ratio of free amino acids) in favourable developmental outcomes, compared to regular formula [10]. The same line of research has previously shown higher satiation among infants fed regular formula with added glutamate compared to regular formula. The authors have clearly framed their observation in light of the role of glutamate (and other free amino acids for that matter) as signalling satiation to the central nervous system [25]. Interestingly, the satiation was equally high when infants were fed extensively hydrolysed formula, which also contains high levels of glutamate and other free amino acids [25]. While dietary glutamate is not considered to enter the brain in relation to the blood-brain barrier, it may indirectly activate brain areas (and conceivably influence brain functions, including ingestive behaviours) since it is sensed in the oral cavity and the intestine [26; 27]. Thus, it may have the capacity to transfer information to the central nervous system through the vagal afferent system [26; 27]. Moreover, animal studies suggest that administration of monosodium glutamate directly affects several behavioural aspects and cognitive capacities and the findings are mixed, also depending on age of assessment [28; 29]. Thus, the free amino acid content, in particular glutamate, of hydrolysed formulas (extensive and partial forms) compared to regular ones may provide a possible mechanistic explanation of their associations with neurodevelopment among a small sample of term infants [10].

**Aim**

The aim of the present study is to examine the associations between the degree of protein hydrolysis in infant formula and child neurodevelopment up to 3.5 years of age, among formula-fed infants. We hypothesise that the early consumption of hydrolysed formulas predicts more favourable neurodevelopmental outcomes within the first four years of life, with stronger associations for formulas with extensive hydrolysates than for those with partial hydrolysates.

## Materials and Methods

### Study population

The present analyses were based on data from the French ELFE study, a nationwide birth cohort, which included 18329 infants born in 2011 in a random sample of 349 maternity wards around metropolitan France across four recruitment waves [30]. Inclusion criteria included singleton or twin births, term and moderate to late preterm births (≥33 gestational weeks), mother aged ≥18 years old, and no plan to move outside metropolitan France within the next 3 years.

### Ethical approval

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the Advisory Committee for the Treatment of Information on Health Research (Comité Consultatif sur le Traitement des Informations pour la Recherche en Santé/ File number 10.623), the National Agency Regulating Data Protection (Commission Nationale Informatique et Libertés/ File number 910504) and the National Statistics Council (File number 2011X716AU). Written informed consent was obtained from all subjects/patients. Mothers provided written informed consent for themselves and their children [30]. Fathers could also provide consent if present at the maternity ward; otherwise they were informed afterwards and they could object to their child's participation.

### Infant feeding

Data on milk feeding practices were collected monthly from 2 to 10 months and then at 12 and 24 months. From these data, any breastfeeding duration was calculated as previously described [31]. From 2 to 10 months, the name and brand of the formula was reported for formula-fed infants[7]. According to the label on the formulas defining the degree of protein hydrolysis, formulas were classified as containing non-hydrolysed proteins (nHF), partially hydrolysed proteins (pHF), extensively hydrolysed proteins (eHF), amino acid mixture (AA) [8]. While eHF and AA explicitly address cow milk protein allergy (CMPA) [32], formulas with HA label are, by definition, based on partially hydrolysed proteins. In France, some infant formulas without the HA label (pHF/non-HA) also contained partially hydrolyzed proteins, probably with a lower level of hydrolysis. Thus, we decided to consider them separately. Terms on the label of formulas (ingredients), which facilitated the classification of formulas in terms of protein hydrolysates in the present paper are shown on Supplementary table S1.

Child neurodevelopment

The French version of the Child Development Inventory (CDI) was administered during the phone interviews with parents at 1- and 3.5-years post-partum [30; 33; 34]. At age 1 year, items adapted to the developmental age were selected from the full version of the CDI, while at age 3.5 years, the brief version was used –in particular the two parts highlighting developmental milestones at this age [34]. The CDI-1 assesses six developmental domains (social skills, self-help, gross motor skills, fine motor skills, language expression and language comprehension), and the CDI-3.5 assesses two additional domains (characters and numbers). Response options for each item were yes (1) if the child had achieved the described ability and no (0) if not. The summary score of items in CDI-1 and CDI-3.5 was used to assess overall child neurodevelopment, ranging from 0 to 50 at 1 year and 17 to 62 at 3.5 years (under the assumption that earlier milestones had been reached, thus the minimum score at 3.5 years corresponds to the maximum score of the CDI items for younger ages) [34].

Second, the brief French version of the MacArthur-Bates Communicative Development Inventory was used during phone interviews with parents at 2 years (MB-2) to assess children's early language development on a 100-point continuous score (each point corresponds to a word expressed by the child) [35].

Last, the Pictures Similarities sub-scale from the British Ability Scale was administered by a trained research assistants during home visits at 3.5 years (PS-3.5) to assess child cognitive development in terms of their pictorial reasoning ability [36]. The score ranged from 10 to 119.

Perinatal, family and feeding characteristics

Data on family background characteristics were collected by trained interviewers at the maternity ward and they were complemented by data on the newborn according to medical records [30]. Complementary information regarding the families were obtained during phone interviews at 2 months and 1 year postpartum.

As regards family background characteristics, the following information was of interest: mother's age (<25 years, 25-29 years, 30-34 years, ≥35 years), education (upper secondary or lower, high school diploma, 3-y university education, at least 5-y university education), employment (employed, unemployed, out of the labour force ─i.e. housewife, retired, students), and migration status (migrant/not born to French parents, descendant of at least one migrant parent, majority population/born to French parents), household income per consumption unit (≤1111 €/month, 1112 –1500 €/month, 1501–1944 €/month, 1945 –2500

€/month, >2500 €/month), maternal smoking during pregnancy (never smoker, smoker only before pregnancy, smoker only in early pregnancy, smoker throughout pregnancy), parental history of allergy (no parent with allergy, at least one parent with allergy), sibling history of allergy (no sibling, no sibling with allergy, at least one sibling with allergy), mother's diet quality during the last trimester of pregnancy using the Probability of Adequate Nutrient intake based Diet quality index [PANDiet] score ─adapted for pregnancy, which reflects nutrient-based reference guidelines adapted for pregnancy; total scores range from 0 to 100) [37; 38]. The region of residence (Paris region, North, East, Paris Basin – East, Paris Basin – West, West, Southwest, Southeast, Mediterranean) of the family, as well as the urban/rural area of living, were determined from the postal code of residence. At the 1-year interview, the mother indicated the frequency (rarely/never/sometimes, often) of some activities with their child: playing, reading books, drawing, speaking, tickling/massage [30; 39]. The modal value of these activities was used to estimate a maternal stimulation score indicating a family environment that is conducive to favourable child development [40; 41].

Characteristics related to the infant include the following: sex (boy, girl), gestational age (in weeks), physician consulted between hospital discharge and 2 months post-partum (General practitioner, Paediatrician, Another child doctor, None/other), allergy to cow milk (yes/no). Birth weight was classified into 3 categories (small/adequate/large for gestational age) according to the French Audipog reference curves [42].

Sample selection

The ELFE sample consisted of 18329 infants and their families who fulfilled the inclusion criteria and consented to participate, at least in the beginning (Figure 1). Figure 1 shows the consecutive steps leading to the analytical sample, which was used for the main analyses (complete-case). Additional (sensitivity) analyses accounted for missing data through multiple imputations of the confounding variables. The analyses and the rationale thereof are described in the next section. Varying sample sizes across analyses are due to missing data in the respective neurodevelopmental scores (Figure 1).

Families who withdrew consent (n=57) were excluded from the analyses. For families with twins, we proceeded to a random selection of one twin to avoid clustered data (n=287). Further exclusions were performed in relation to the exposure, i.e. no follow-up at 2 months (n=1696), no formula feeding at 2 months (n=5054), and no information on the degree of protein hydrolysis in formula (n=658). From the remaining sample, infants with missing data across all neurodevelopmental outcomes were further excluded (n=1205) along with those

who had missing data in adjustment variables (n=2393), leading to the analytical sample (n=6979). The analytical sample provided the basis for the complete-case analyses (main models) according to the availability of neurodevelopment data, i.e., at 1 year for CDI (n=6977), at 2 years for MB (n=6145), and at 3.5 years for CDI (n=5696) and for PS (n=4511). Multiple imputations of the missing confounding variables will be described in the next section. These analyses were based on the analytical sample including missing data on confounding variables (n=9372), and they were performed according to data availability on the neurodevelopmental outcomes, on 8980 at 1 year for CDI, 7962 children at 2 years for MB, and 7334 and 5695 at 3.5 years for CDI and for PS respectively.

As compared to children and their families who were included in the analyses, those who were excluded (apart from those who had withdrawn consent and the selection of twins, n=11006) were characterised by slightly lower income levels (mean €1600 vs. €1675 per consumption unit, $p<0.001$), more mothers with a migration history (24.5% vs. 15.7%, $p<0.001$) and with a higher education level (22.5% vs. 17.6% at least 5-y university education, $p<0.001$) and higher rates of mothers' never smoking (59.4% vs. 53.7%, $p<0.001$). On the other hand, excluded sample was similar to the included sample in terms of child sex (girls: 48.6% vs. 48.4%, $p=0.80$), mean gestational age (39.2 vs. 39.2 weeks, $p=0.06$), and mean maternal age (30.7 vs. 30.9 years old, $p=0.07$).

Statistical analyses

For the total analytical sample, frequencies (n) and means (SD) were computed.

We considered the following neurodevelopmental outcomes: one summary and six domain-specific scores for child motor and cognitive development were based on the CDI at 1- and 3.5-years post-partum, a score for early language development was based on the brief French MacArthur-Bates Inventory at 2 years post-partum (MB-2), and a score for pictorial reasoning ability was additionally assessed according to the PS sub-scale at 3.5-years post-partum (PS-3.5).

The six domain-specific sub scores of the CDI at 1- and 3.5-years did not follow a normal distribution, thus they were divided into quartiles. Children within the lowest quartile were considered as having a poor developmental sub-score and they were compared to children from the three upper quartiles (reference group). The overall CDI scores as well as the MacArthur-Bates scores and the Picture Similarities score were considered as continuous variables.

Binary logistic and linear regression models were used to conduct the unadjusted analyses between the degree of protein hydrolysis in infant formula and neurodevelopmental outcomes. Multivariable logistic and linear regression models were run to account for confounding factors. These were identified from the literature and selected using the directed acyclic graph method [43]. Then multivariable models were adjusted for: study design variables (maternity size and recruitment wave), socio-demographic and family characteristics (parental stimulation, maternal age, maternal employment, maternal educational attainment, migration history, household income, region of residence, urban/rural area), infant characteristics (child sex), perinatal and health-related factors (gestational age in weeks, gestational age, parents' and siblings' history of allergies, type of physician consulted between hospital discharge and 2 months of age, any breastfeeding duration, cow-milk protein allergy (CMPA) reported at the 2-month interview), lifestyle factors (maternal smoking during pregnancy, dietary quality using a validated scoring system adapted for the French population and to nutritional needs during pregnancy). In addition, all models were adjusted for the child's age (in months) at the time of the respective neurodevelopmental assessments.

The main analyses were conducted on the complete-case sample. Sensitivity analyses were performed using additional models for sub-samples of infants without any congenital malformations (n=6713) and term infants (n=6604). These sub-samples were excluded because of the clear links between these birth outcomes and later neurodevelopmental outcomes [44; 45]. Additional sensitivity analyses included sub-samples of infants who did not change formula over the first 2 months' follow-up (n=3680) and those who did not change formula between 2 and 6 months (n=3970). Based on infants with complete data on infant formula consumption between 2 and 6 months (at 2 months n=4063 for nHF; n=118 for pHF/non-HA; n=190 for pHF/HA; n=42 for eHF/AA), an overwhelming 93% of infants consuming nHF at 2 months showed a consistent consumption of this formula between 2 and 6 months. By contrast, one out of two infants consuming a hydrolysed formula (of any type according to the classification in the present paper) at 2 months showed an inconsistent use of it between 2 and 6 months (45.8%, 51%, and 45.2% for pHF/non-HA, pHF/HA and eHF/AA, respectively).

To deal with selection and attrition bias, a sensitivity analysis was conducted with weighted data on the complete-case sample. Weighting was calculated to take into account the inclusion procedure and biases related to non-consent or attrition and also included calibration on margins from the state register's statistical data and the 2010 French National Perinatal study

[46] on the following variables: age, region, marital status, migration status, level of education, and primiparity (https://www.elfe-france.fr/fichier/rte/178/Coté%20recherche/Weighting-Elfe-surveys-general-document.pdf). A specific weighting was calculated for the sub-samples included in the complete-case analyses at 1 and 3.5-year follow-ups, respectively.

Finally, a sensitivity analysis was performed with multiple imputation of confounding variables to deal with missing data [47]. This approach has been integral to the analytical plan of the ELFE study –a nationwide birth cohort with long follow-up [30]– and it has been applied in multiple analyses in order to address the bias introduced due to missing data [39; 48; 49; 50]. Based on the assumption that confounding variables were missing at random and using the fully conditional specification method, the procedure of multiple imputations generated five independent and complete data sets (SAS software: MI procedure, FCS statement, NIMPUTE option). Pooled effect estimates were then calculated for each outcome of interest (SAS software: MIANALYSE procedure). For significance testing of categorical variables, the median of the P values from the imputed data analyses in each data set was used [51].

All analyses were carried out using SAS v9.4 (SAS Institute Inc., Cary, NC). Significance was set at $p<0.05$.

**Results**

Table 1 summarises infant and family characteristics according to the degree of protein hydrolysis of the formula in the analytical sample (n=6979). The majority of infants (n=6432) consumed nHF, and the rest consumed increasingly hydrolysed formulas as follows: pHF/non-HA (n=189), pHF/HA (n=288), and eHF/AA (n=70). The majority of infants consuming eHF/AA had CMPA at 2 months. By contrast, infants fed nHF had higher rates of no parental family history for allergies. Overall, summary and sub-domain neurodevelopmental scores were found to be similar across formula groups with an increasing degree of protein hydrolysis (Table 2).

The degree of protein hydrolysis of the formula fed at 2 months was not related to the overall neurodevelopmental scores from 1 to 3.5 years, in the main analyses (complete-case adjusted) and those adjusted after multiple imputations (Table 3 and Supplementary table S4). When the specific weighting was applied to account for selection and attrition bias, compared to infants having consumed nHF at 2 months, infants having consumed pHF/non-HA had lower CDI-1 score, whereas infants having consumed eHF/AA had higher CDI-3.5 score (Table 3).

When considering the specific developmental domains separately, the degree of protein hydrolysis in the 2-month infant formula was not related with the risk of having a poor score on social skills, self-help, fine motor skills, language expression and language comprehension, at the ages of 1 or 3.5 years, in the main analyses (complete-case adjusted), shown in Table 4.

At the age of 1 year, compared to children having consumed a non-hydrolysed formula, those having consumed pHF/HA were more likely to have a poor score on the gross motor sub-scale (Table 4). While these findings at the 1-year follow-up were consistent for the adjusted main analyses and in the analyses after multiple imputations, they did not reach significance after weighting, though the trend for effects remained the same (Table 4 and Supplementary table S5).

At the age of 3.5 years, early consumption of pHF/non-HA at 2 months was associated with a lower risk of having poor social skills, compared to having consumed non-hydrolysed formula (Table 4). This finding was also shown in the analyses with multiple imputations and in the weighted analyses, while children having consumed eHF/AA were less likely to have a poor score on fine motor skills in the weighted analyses only (Table 4).

The findings of the main analyses (complete-case) were in line with the unadjusted analyses (Supplementary tables S2 and S3) and the sensitivity analyses including specific sub-samples, except for the analyses at 3.5 years including infants without congenital malformations which were in line with the weighted analyses (Supplementary tables S4 and S5).

## Discussion

The present study is the first to examine the effects of the use of formula with varying degrees of protein hydrolysis on child neurodevelopment up to 3.5 years of age in a birth cohort. The degree of protein hydrolysis in infant formula consumed at 2 months of age was not related to overall neurodevelopmental scores up to 3.5 years. Some associations were found with the gross motor skills CDI sub-domain but they were not consistent at 1 and 3.5 years as well as across all sensitivity analyses (including specific sub-samples and also accounting for attrition and selection bias through weighted data as well as addressing missing data through multiple imputation procedures). Nonetheless, associations were only shown for formulas with partial hydrolysates and they were not extended to those with extensive hydrolysates.

As expected, the use of hydrolysed formulas, in particular the extensively hydrolysed ones, aligned with the presence of cow's milk protein allergy and a family history of allergy. Such findings reflect current recommendations and/or common practices regarding the use of hydrolysed formulas [32; 52; 53]. There is scarce evidence regarding long term effects (at least over 1 year of age) on child neurodevelopment of the use of infant formula in infancy. Therefore, we cannot directly compare our findings to previous studies. Our findings do not confirm the hypotheses by Mennella *et al.* [10], who have provided preliminary evidence on certain favourable effects of formula with extensively hydrolysed proteins on motor skills and cognition among infants younger than the age of 1 year over 8 months follow-up. In addition, our findings do not support stronger associations according to an increasing degree of protein hydrolysis; we observed unfavourable associations with gross motor skills with partially hydrolysed formula only. Yet, the observed associations were transient, i.e. they were present at 1 year of age but they were not significant anymore at the 3.5-years follow-up. This may support the argument of transient neurodevelopmental effects of formula in early life which was also presented by Mennella *et al.* [10] according to monthly assessment at a younger age than in the present study (i.e. between 5.5 and 8.5 months of age). Further evidence on transient effects may relate to the free amino acid content, especially glutamate, which marks an important difference between hydrolysed protein formulas from the regular ones [25]. In particular, an animal study involving rats fed monosodium glutamate during the neonatal period found transient effects of the use of monosodium glutamate on locomotor activity whereby at 3 weeks of age there was an increase in locomotor activity which was followed by a marked hypoactivity the week after [28]. These follow-up times in the animal study roughly correspond to those examined in our study [54]. Taken together, these findings highlight the relevance of length and timing of follow-up across studies due to the high neuroplasticity during the early life stages [55].

It is conceivable that the literature on infant formula with hydrolysed proteins focuses on the free amino acid content of these formulas. Free amino acids are also present in human milk at higher concentrations than regular formula and they could explain some of the differences in developmental indicators between breastfeeding and formula feeding [21; 25; 56; 57]. However, drawing parallels between the high content in free amino acids (or any other biological component for that matter, such as biologically active molecules, microbiota, etc. etc.) of hydrolysed formulas and breast milk, may fail to account for other aspects of breastfeeding that may promote cognitive development among children, such as infant attachment parenting

practices and the home environment [58; 59; 60; 61]. For example, McCormick *et al.* [62] showed that while aspects of the home environment along with certain nutrients did differentiate between the identified child cognitive trajectories (i.e. consistently high scores, increasing scores, intermediate scores with early and late decline, and consistently low scores), exclusive breastfeeding had limited discriminatory power in relation to cognitive development.

Strengths and limitations

ELFE is a large birth cohort in France. Its prospective design limits recall bias for both exposure and outcome assessments. The very large sample and the collection of detailed socio-demographic or economic data ensure good statistical power and favor control for potential confounders, although residual confounding may remain. Of note, indicators for developmental delays in early infancy were not considered. Developmental outcomes may indirectly relate to the choice of infant formula since limited tolerance to standard feeds and regurgitation, which appear more common among children with developmental delays [63; 64], may have prompted the use of hydrolysed formulas in infancy [7; 52]. Moreover, due to the small size (n=1) of the analytical sample consuming elemental formula, it was collapsed with the most similar category in terms of free amino acid content namely extensively hydrolysed formula (n=69). In fact, the use of any type of formulas with protein hydrolysates was not very prevalent; the highest prevalence was registered for the use of partially hydrolysed forms with hypoallergenic label (just over 4%). Although these findings are based on data from a nationwide cohort and they do map the use of formulas in France, large samples for the study of hydrolysed formulas have not been available [7]. Thus, our analyses did not have the capacity to distinguish between extensively hydrolysed and elemental formulas, and they were generally limited by the low statistical power as per the groups of high degree of protein hydrolysis. Finally, the sample considered for the present analyses was based on a higher rate of privileged families than the initial ELFE sample, which could limit the generalization of our results [30]. However, sensitivity analyses based on weighted data, accounting for selection and attrition biases, gave similar findings, suggesting that this bias had limited impact on our conclusions. Similarly, missing data can introduce bias, yet analyses based on imputed data yielded similar findings. Moreover, diverse ranges of brands for the same type of hydrolysed infant formulas were used by families, and changes in infant formula were frequent. Sensitivity analyses including infants who had not changed infant formula for up to 2 months follow-up and those who did not change the type of formula between 2 and 6 months, pointed to similar conclusions. Regarding glutamate, which is implicated in a mechanistic explanation

of the initial hypothesis, the glutamate content of indicated formulas was not assessed. Using parental questionnaires may have introduced biases, including social desirability bias and imprecision, but parents completed a battery of valid and reliable instruments [34; 35; 36] to allow for international comparisons and reduce the above-mentioned biases. Still, the on-site assessment of the Picture Similarities test (as part of the BAS) by trained research assistants showed a similar pattern of (no) association.

## Conclusion

In summary, among formula-fed infants, the degree of protein hydrolysis in infant formula fed at 2 months was not associated with overall neurodevelopmental scores up to 3.5 years of age. These findings are in favour of the safety of use of such formulas, beyond growth trajectories [53; 65; 66]. However, it would be important to replicate these analyses across settings with a different distribution of the studied formulas, as well as in more vulnerable populations.

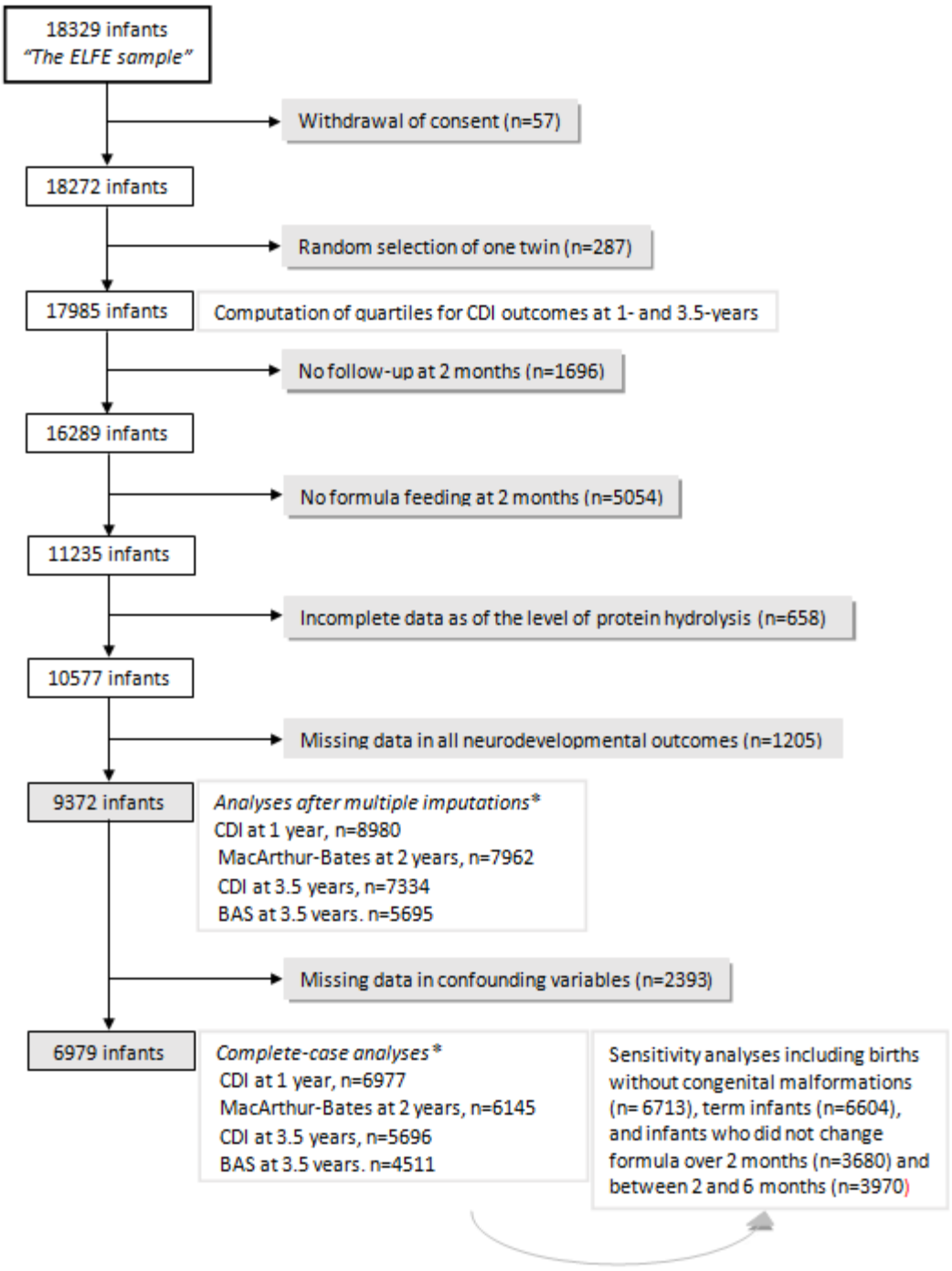


**Figure 1.** Flowchart for the analyses. ⁎Varying sample sizes due to missing data in the respective neurodevelopmental score

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## Conflicts of interest

The authors had no conflicts of interest relevant to this article to disclose.

## Authors' contributions

M.S. conceptualised the study, conducted the formal analyses and drafted the manuscript. B.L-G. and S.N. conceptualised and designed the study, designed instruments for nutritional data, supervised data collection and management, contributed to the interpretation of the findings and critically reviewed the manuscript. J.Y.B. and M.T. managed data on neurodevelopment and critically reviewed the manuscript. M-A.C. coordinated the study and reviewed the manuscript.

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**Table 1.** Sample characteristics according to the degree of protein hydrolysis in infant formula consumed at 2 months (n=6979)

| | **Non hydrolyzed formula (nHF)** | **Partially hydrolysed formula without HA label (pHF/non-HA)** | **Partially hydrolysed formula with HA label (pHF/HA)** | **Extensively hydrolysed formula or amino acids mixture (eHF/AA)** |
|---|---|---|---|---|
| N (%) | 6432 (92.2%) | 189 (2.71%) | 288 (4.1%) | 70 (1%) |
| **MATERNAL/FAMILY CHARACTERISTICS** | | | | |
| **Maternal age, %(n)** | | | | |
| <25 years | 8.8% (567) | 6.3% (12) | 5.9% (17) | 11.4% (8) |
| 25-29 years | 31.6% (2035) | 35.4% (67) | 29.2% (84) | 21.4% (15) |
| 30-34 years | 37.2% (2391) | 33.3% (63) | 41% (118) | 44.3% (31) |
| ≥35 years | 22.4% (1439) | 24.9% (47) | 24% (69) | 22.9% (16) |
| **Maternal employment, %(n)** | | | | |
| Employed | 76.8% (4940) | 86.2% (163) | 85.4% (246) | 78.6% (55) |
| Unemployed | 11.3% (729) | 7.4% (14) | 5.9% (17) | 17.1% (12) |
| Out of the labour force (i.e. housewife, retired, students) | 11.9% (763) | 6.3% (12) | 8.7% (25) | 4.3% (3) |
| **Maternal education, %(n)** | | | | |
| Upper secondary or lower | 40.3% (2594) | 37.6% (71) | 28.1% (81) | 32.9% (23) |

| | | | | |
|---|---|---|---|---|
| Intermediate | 25.3% (1626) | 25.9% (49) | 27.4% (79) | 24.3% (17) |
| 3-y university degree | 17.1% (1102) | 18% (34) | 21.5% (62) | 22.9% (16) |
| At least 5-y university degree | 17.3% (1110) | 18.5% (35) | 22.9% (66) | 20% (14) |
| **Maternal migration history, %(n)** | | | | |
| Immigrant | 6.1% (390) | 4.8% (9) | 4.2% (12) | 4.3% (3) |
| Descendant of at least one immigrant | 9.7% (626) | 12.2% (23) | 7.6% (22) | 11.4% (8) |
| Rest of population | 84.2% (5416) | 83.1% (157) | 88.2% (254) | 84.3% (59) |
| **Household income, %(n)** | | | | |
| ≤ 1111 €/month | 19.6% (1258) | 15.9% (30) | 11.5% (33) | 17.1% (12) |
| 1112 - 1500 €/month | 29.5% (1898) | 29.6% (56) | 29.2% (84) | 30% (21) |
| 1501 - 1944 €/month | 25.8% (1658) | 28% (53) | 26.4% (76) | 37.1% (26) |
| 1945 - 2500 €/month | 15.8% (1018) | 16.9% (32) | 18.4% (53) | 12.9% (9) |
| > 2500 €/month | 9.3% (600) | 9.5% (18) | 14.6% (42) | 2.9% (2) |
| **Residence area, %(n)** | | | | |
| Rural | 25.3% (1630) | 23.8% (45) | 24.3% (70) | 30% (21) |
| Urban | 74.7% (4802) | 76.2% (144) | 75.7% (218) | 70% (49) |
| **Diet quality during pregnancy, Mean (SD)** | 54.8 (9) | 55 (9.2) | 54.7 (9.4) | 55.9 (9.7) |
| **Mother smoking during pregnancy, %(n)** | | | | |
| Never smoker | 53.5% (3441) | 55% (104) | 56.3% (162) | 54.3% (38) |

| | | | | |
|---|---|---|---|---|
| Smoker only before pregnancy | 25.5% (1642) | 28% (53) | 25% (72) | 27.1% (19) |
| Smoker only in early pregnancy | 4% (255) | 4.2% (8) | 5.9% (17) | 7.1% (5) |
| Smoker throughout pregnancy | 17% (1094) | 12.7% (24) | 12.8% (37) | 11.4% (8) |
| **Parental stimulation [*], %(n)** | | | | |
| Often | 66.1% (4250) | 66.1% (125) | 73.3% (211) | 65.7% (46) |
| Sometimes/Rarely/Never | 33.9% (2182) | 33.9% (64) | 26.7% (77) | 34.3% (24) |
| **Parents' history of allergy, %(n)** | | | | |
| No parent with allergy | 50.4% (3243) | 41.3% (78) | 31.9% (92) | 45.7% (32) |
| At least one parent with allergy | 49.6% (3189) | 58.7% (111) | 68.1% (196) | 54.3% (38) |
| **Sibling history of allergy, %(n)** | | | | |
| No sibling | 46.4% (2984) | 45.5% (86) | 50% (144) | 44.3% (31) |
| No sibling with allergy | 41% (2637) | 39.2% (74) | 30.6% (88) | 37.1% (26) |
| At least one sibling with allergy | 12.6% (811) | 15.3% (29) | 19.4% (56) | 18.6% (13) |
| **NEWBORN/INFANT CHARACTERISTICS** | | | | |
| **Child sex, %(n)** | | | | |
| Boy | 51.1% (3287) | 54% (102) | 60.1% (173) | 51.4% (36) |
| Girl | 48.9% (3145) | 46% (87) | 39.9% (115) | 48.6% (34) |
| **Birth weight category [†], %(n)** | | | | |
| Small for GA | 9.7% (624) | 8.5% (16) | 13.2% (38) | 11.4% (8) |
| Adequate for GA | 80.3% (5166) | 79.4% (150) | 77.1% (222) | 81.4% (57) |

| | | | | |
|---|---|---|---|---|
| Large for GA | 10% (642) | 12.2% (23) | 9.7% (28) | 7.1% (5) |
| **Infant CMPA at 2 months, %(n)** | | | | |
| Yes | 1% (62) | 6.9% (13) | 1.4% (4) | 61.4% (43) |
| No | 99% (6370) | 93.1% (176) | 98.6% (284) | 38.6% (27) |
| **Gestational age in weeks, Mean (SD)** | 39.2(1.5) | 39.1(1.6) | 39.3(1.4) | 39.3(1.5) |
| **Physician consulted between hospital discharge and 2 months, %(n)** | | | | |
| General practitioner | 46.8% (3013) | 34.9% (66) | 46.2% (133) | 32.9% (23) |
| Paediatrician | 36.1% (2320) | 44.4% (84) | 37.5% (108) | 38.6% (27) |
| Another child doctor ‡ | 11.9% (763) | 13.2% (25) | 10.1% (29) | 15.7% (11) |
| None/other | 5.2% (336) | 7.4% (14) | 6.3% (18) | 12.9% (9) |
| **INFANT DIET (BREASTFEEDING DURATION)** | | | | |
| **Any breastfeeding duration in months, Mean (SD)** | 1.3(2.5) | 1.1(1.7) | 1.6(2.1) | 0.9(1.5) |

HA label, hypoallergenic label; GA, gestational age; CMPA, Cow-milk protein allergy

* Parental stimulation was defined according to the frequency of activities (e.g. drawing, playing) with the child, as reported by mothers at 1year follow-up

† Size at gestational age is classified according to birth weight

‡ From maternity unit or from child and maternal protection centres

**Table 2.** Neurodevelopmental scores across infant formulas with an increasing degree of protein hydrolysis (n=6979)

| | **Neurodevelopmental scores** | | | | |
|---|---|---|---|---|---|
| | **Non hydrolyzed formula (nHF)** | **Partially hydrolysed formula without HA label (pHF/non-HA)** | **Partially hydrolysed formula with HA label (pHF/HA)** | **Extensively hydrolysed formula or amino acids mixture (eHF/AA)** | **p-value** |
| **Child Developmental Inventory (CDI-1)-summary score, mean (SD) [range: 0-50]** | 36.7 (5.5) | 36 (5.5) | 36.5 (5.6) | 36.5 (5.4) | 0.30 |
| **High risk in the sub-domain scores for Child Developmental Inventory (CDI-1), % (n)** | | | | | |
| Social skills ⁎ | 17.6% (1133) | 19% (36) | 18.8% (54) | 14.3% (10) | 0.80 |
| Self-help † | 10.5% (675) | 14.3% (27) | 11.1% (32) | 15.7% (11) | 0.20 |
| Gross motor skills ‡ | 18.4% (1185) | 21.2% (40) | 25.3% (73) | 18.6% (13) | 0.02 |
| Fine motor skills § | 25.2% (1621) | 27% (51) | 28.5% (82) | 22.9% (16) | 0.60 |
| Expressive language ‖ | 16.1% (1038) | 19% (36) | 13.9% (40) | 17.1% (12) | 0.50 |
| Receptive language ¶ | 18.7% (1200) | 20.6% (39) | 16.7% (48) | 10% (7) | 0.20 |
| **MacArthur-Bates Communicative Development Inventory (MB-2), mean (SD)** | 71.9 (25) | 70.4 (27.2) | 74.2 (24.7) | 72.5 (20.7) | 0.50 |

| | | | | | |
|---|---|---|---|---|---|
| **[range: 0-100]** | | | | | |
| **Child Developmental Inventory (CDI-3.5)-summary score, mean (SD) [range: 17-62]** | 53.5 (5.2) | 53.9 (4.7) | 53 (5.7) | 53.3 (4.9) | 0.40 |
| **High risk in the sub-domain scores for Child Developmental Inventory (CDI-3), % (n)** | | | | | |
| Social skills ⁎ | 15.1% (792) | 9.5% (15) | 14.1% (34) | 13.6% (8) | 0.30 |
| Self-help † | 12.1% (636) | 15.8% (25) | 12.9% (31) | 15.3% (9) | 0.50 |
| Gross motor skills ‡ | 10.7% (558) | 12% (19) | 11.2% (27) | 8.5% (5) | 0.90 |
| Fine motor skills § | 20.6% (1081) | 24.1% (38) | 23.2% (56) | 13.6% (8) | 0.30 |
| Expressive language ‖ | 17.9% (935) | 13.3% (21) | 18.7% (45) | 23.7% (14) | 0.30 |
| Receptive language ¶ | 12.1% (636) | 15.8% (25) | 12.9% (31) | 15.3% (9) | 0.50 |
| **Picture Similarities sub-scale (PS-3.5), mean (SD) [range: 10-119]** | 63.9 (29.3) | 63.9 (28.7) | 64.5 (28) | 64.2 (32.2) | 0.70 |

high risk score (<25$^{th}$ percentile) ⁎ at 1-year is <6 and at 3.5 years is <9; † at 1-year is <4 and at 3.5 years is <7; ‡ at 1-year is <3 and at 3.5 years is <8; § at 1-year is <7 and at 3.5 years is <6; ‖ at 1-year is <5 and at 3.5 years is <9; ¶ at 1-year is <7 and at 3.5 years is <8.

Varying sample sizes due to missing data in the respective neurodevelopmental score.

**Table 3.** Adjusted estimates of summary developmental scores at 1, 2 and 3.5 years across formulas with increasing degree of protein hydrolysis consumed at 2 months, complete-case analyses

| | Summary developmental scores (on a continuous scale) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | N | CDI-1 | N | MB-2 | N | CDI-3.5 | N | PS-3.5 |
| *Main analysis* | *6977* | | *6145* | | *5696* | | *4511* | |
| nHF | 6430 | 0.00 [Ref] | 5649 | 0.00 [Ref] | 5238 | 0.00 [Ref] | 4146 | 0.00 [Ref] |
| pHF/non-HA | 189 | -0.70 [-1.44; 0.05] | 175 | -1.33 [-4.93; 2.26] | 158 | 0.41 [-0.37; 1.19] | 127 | -0.34 [-3.07; 2.40] |
| pHF/HA | 288 | -0.22 [-0.83; 0.39] | 258 | 2.02 [-0.98; 5.01] | 241 | -0.38 [-1.02; 0.26] | 196 | 0.19 [-2.04; 2.42] |
| eHF/AA | 70 | -0.27 [-1.63; 1.08] | 63 | 2.37 [-4.34; 9.08] | 59 | 0.77 [-0.66; 2.21] | 42 | -0.67 [-5.86; 4.52] |
| *Weighted analyses* † | *6976* | | *6145* | | *5695* | | *4511* | |
| nHF | 6429 | 0.00 [Ref] | 5649 | 0.00 [Ref] | 5237 | 0.00 [Ref] | 4146 | 0.00 [Ref] |
| pHF/non-HA | 189 | -1.09 [-1.94; -0.24] | 175 | -3.78 [-9.12; 1.55] | 158 | 0.63 [-0.14; 1.40] | 127 | -0.75 [-3.71; 2.21] |
| pHF/HA | 288 | 0.16 [-0.62; 0.93] | 258 | 3.52 [0.00; 7.04] | 241 | 0.00 [-0.82; 0.82] | 196 | -1.57 [-4.70; 1.57] |
| eHF/AA | 70 | -0.44 [-1.74; 0.87] | 63 | 5.67 [-2.91; 14.25] | 59 | 1.74 [0.04; 3.45] | 42 | -0.07 [-4.55; 4.41] |

CDI-1: 1-year Child Development Inventory; MB-2: 2-year MacArthur-Bates Communicative Development Inventory;

CDI-3.5: 3.5-year Child Development Inventory; PS-3.5: 3.5-year Picture Similarities ability score from the British Ability Scale.

nHF, non-hydrolysed formula; pHF/non-HA, partially hydrolysed formula without any hypoallergenic label;

pHF/HA, partially hydrolysed formula with a hypoallergenic label;

eHF/AA, extensively hydrolysed formula, or formula based on amino-acids.

Values are estimates [95% CI] from linear regression models adjusted for child age at each assessment, mother's age, education, employment, and migration status, household income, maternal smoking during pregnancy, parental and sibling history of allergy, mother's dietary quality during pregnancy, urban/rural are of living, region of residence, parental stimulation, child sex, gestational age, and size according to gestational age, physician consulted between hospital discharge and 2 months post-partum, allergy to cow milk, any breastfeeding duration.

† Estimates are adjusted for the aforementioned factors and are weighted in order to account for the inclusion procedure and biases related to non-consent or attrition.

Varying sample sizes due to missing data in the respective neurodevelopmental score.

**Table 4.** Adjusted odds-ratios (ORs) of having a poor developmental sub-score across formulas with increasing degree of protein hydrolysis consumed at 2 months, complete-case analyses at 1-year follow-up (n=6977) and at 3.5 years follow-up (n=5696)

| | **Poor developmental sub-score (lowest quartile vs three other quartiles)** | | | | | |
|---|---|---|---|---|---|---|
| | Social skills | Self-help | Gross motor skills | Fine motor skills | Language expression | Language comprehension |
| **1-year follow-up** | | | | | | |
| *Main analysis* | | | | | | |
| nHF | 1.00 [Ref] | 1.00 [Ref] | 1.00 [Ref] | 1.00 [Ref] | 1.00 [Ref] | 1.00 [Ref] |
| pHF/non-HA | 1.10 [0.75; 1.61] | 1.41 [0.92; 2.16] | 1.22 [0.85; 1.76] | 1.11 [0.79; 1.56] | 1.18 [0.80; 1.72] | 1.15 [0.79; 1.67] |
| pHF/HA | 1.09 [0.79; 1.49] | 1.03 [0.70; 1.51] | 1.60 [1.20; 2.12] | 1.22 [0.93; 1.60] | 0.84 [0.59; 1.19] | 0.90 [0.65; 1.25] |
| eHF/AA | 0.73 [0.34; 1.59] | 1.48 [0.68; 3.22] | 1.03 [0.51; 2.08] | 1.04 [0.55; 1.99] | 0.95 [0.45; 1.98] | 0.46 [0.19; 1.08] |
| *Weighted analyses* | | | | | | |
| nHF | 1.00 [Ref] | 1.00 [Ref] | 1.00 [Ref] | 1.00 [Ref] | 1.00 [Ref] | 1.00 [Ref] |
| pHF/non-HA | 1.07 [0.68; 1.69] | 1.20 [0.70; 2.04] | 1.56 [0.97; 2.52] | 1.22 [0.80; 1.86] | 1.19 [0.70; 2.03] | 1.28 [0.80; 2.03] |
| pHF/HA | 1.08 [0.74; 1.58] | 1.01 [0.63; 1.62] | 1.30 [0.93; 1.81] | 1.04 [0.75; 1.45] | 0.78 [0.49; 1.25] | 0.92 [0.62; 1.38] |
| eHF/AA | 0.63 [0.29; 1.35] | 1.19 [0.48; 2.97] | 0.93 [0.42; 2.04] | 1.52 [0.77; 2.99] | 0.98 [0.39; 2.46] | 0.43 [0.17; 1.13] |
| **3.5-year follow-up** | | | | | | |
| *Main analysis* | | | | | | |
| nHF | 1.00 [Ref] | 1.00 [Ref] | 1.00 [Ref] | 1.00 [Ref] | 1.00 [Ref] | 1.00 [Ref] |
| pHF/non-HA | 0.57 [0.33; 0.99] | 1.27 [0.81; 2.00] | 1.22 [0.74; 2.00] | 1.18 [0.80; 1.75] | 0.70 [0.44; 1.13] | 0.70 [0.43; 1.16] |
| pHF/HA | 0.98 [0.67; 1.44] | 0.95 [0.64; 1.42] | 1.08 [0.71; 1.65] | 1.13 [0.81; 1.56] | 1.12 [0.80; 1.59] | 1.03 [0.72; 1.49] |

| | | | | | | |
|---|---|---|---|---|---|---|
| eHF/AA | 0.82 [0.35; 1.93] | 1.15 [0.49; 2.67] | 0.70 [0.25; 2.00] | 0.43 [0.18; 1.02] | 1.09 [0.53; 2.24] | 0.73 [0.32; 1.66] |
| *Weighted analyses* | | | | | | |
| nHF | 1.00 [Ref] | 1.00 [Ref] | 1.00 [Ref] | 1.00 [Ref] | 1.00 [Ref] | 1.00 [Ref] |
| pHF/non-HA | 0.40 [0.21; 0.77] | 1.39 [0.74; 2.59] | 1.00 [0.55; 1.81] | 0.87 [0.52; 1.45] | 0.72 [0.38; 1.40] | 0.66 [0.31; 1.41] |
| pHF/HA | 0.80 [0.50; 1.29] | 0.76 [0.48; 1.21] | 0.77 [0.47; 1.26] | 1.17 [0.79; 1.74] | 1.26 [0.81; 1.97] | 0.87 [0.54; 1.41] |
| eHF/AA | 0.85 [0.33; 2.17] | 0.95 [0.33; 2.74] | 0.52 [0.19; 1.39] | 0.29 [0.12; 0.73] | 0.77 [0.30; 2.03] | 0.54 [0.23; 1.30] |

nHF, non-hydrolysed formula; pHF/non-HA, partially hydrolysed formula without any hypoallergenic label;

pHF/HA, partially hydrolysed formula with a hypoallergenic label;

eHF/AA, extensively hydrolysed formula, or formula based on amino-acids.

Values are odds-ratios [95% CI] from logistic regression models adjusted for child age at each assessment, mother's age, education, employment, and migration status, household income, maternal smoking during pregnancy, parental and sibling history of allergy, mother's dietary quality during pregnancy, urban/rural are of living, region of residence, parental stimulation, child sex, gestational age, and size according to gestational age, physician consulted between hospital discharge and 2 months post-partum, allergy to cow milk, any breastfeeding duration.

[†] Estimates are adjusted for the aforementioned factors and are weighted in order to account for the inclusion procedure and biases related to non-consent or attrition.

Varying sample sizes due to missing data in the respective neurodevelopmental score.