HAROKOPIO UNIVERSITY

School of Health Sciences & Education
Department of Nutrition and Dietetics
Postgraduate Program “Applied Nutrition and Dietetics”
Discipline: Clinical Nutrition

Effect of type of feeding on the development of allergic manifestations during the first six months of life: The Allergy Reduction Trial.

Master Thesis

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ΧΑΡΟΚΟΠΕΙΟ ΠΑΝΕΠΙΣΤΗΜΙΟ

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Η επίδραση του τρόπου σίτισης στην εκδήλωση αλλεργικών αντιδράσεων κατά τους πρώτους 6 μήνες ζωής: Η μελέτη Allergy Reduction Trial

Μεταπτυχιακή Εργασία

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ΠΕΡΙΛΗΨΗ

Εισαγωγή: Ο επιπολασμός της αλλεργίας στην πρωτεΐνη του αγελαδινού γάλακτος παρατηρείται αυξημένος στις ανεπτυγμένες χώρες τις τελευταίες τέσσερις δεκαετίες, ιδιαίτερα σε παιδιά ηλικίας κάτω των 6 ετών. Επιπλέον, η παρουσία οικογενειακού ιστορικού ατοπίας και ιδιαίτερα της ατοπικής δερματίτιδας, έχουν χαρακτηριστεί ως παράγοντες κινδύνου για την εμφάνιση αλλεργίας στην πρωτεΐνη του αγελαδινού γάλακτος. Η σίτιση των βρεφών με φόρμουλα η οποία αποτελείται από μερικώς υδρολυμένη πρωτεΐνη, έχει προταθεί από την επιστημονική κοινότητα ως μέθοδος την πρόληψης για την ανάπτυξη αλλεργικών εκδηλώσεων σε βρέφη υψηλού κινδύνου που δεν θηλάζουν αποκλειστικά. Ωστόσο, επιπλέον ισχυρά επιστημονικά δεδομένα τα οποία θα υποστηρίζουν την αποτελεσματικότητά της παραπάνω μεθόδου κρίνονται απαραίτητα.

Σκοπός: Ο σκοπός της παρούσας μελέτης είναι η σύγκριση της επίδρασης του αποκλειστικού μητρικού θηλασμού έναντι του μεικτού τρόπου σίτισης (μητρικός θηλασμός και φόρμουλα ή μητρικός θηλασμός και μερικώς υδρολυμένη φόρμουλα) στην ανάπτυξη αλλεργίας στην πρωτεΐνη του αγελαδινού γάλακτος, στην ανάπτυξη ατοπικής δερματίτιδας και στις παραμέτρους ανάπτυξης σε βρέφη υψηλού κινδύνου κατά τους πρώτους 6 μήνες της ζωής.

Μεθοδολογία: Η μελέτη Allergy Reduction Trial – ART αποτελεί μια πολυκεντρική, τυχαιοποιημένη, παράλληλη, διπλά-τυφλή κλινική δοκιμή. Τα βρέφη που συμμετείχαν στη μελέτη είτε θήλαζαν αποκλειστικά, είτε τυχαιοποιήθηκαν σε μια από τις δύο ομάδες: α) μητρικός θηλασμός & μερικώς υδρολυμένη φόρμουλα, β) μητρικός θηλασμός & φόρμουλα άθικτης πρωτεΐνης. Η μελέτη πραγματοποιήθηκε κατά τα έτη 2017-2019. Η επίπτωση και ο σχετικός κίνδυνος υπολογίστηκαν για την αξιολόγηση της εμφάνισης αλλεργίας στην πρωτεΐνη του αγελαδινού γάλακτος και της ατοπικής δερματίτιδας κατά τους πρώτους 6 μήνες της ζωής.

Αποτελέσματα: Μετά από 6 μήνες παρέμβασης, η επίπτωση της ατοπικής δερματίτιδας ήταν υψηλότερη στα βρέφη που θήλαζαν αποκλειστικά σε σύγκριση με την ομάδα που παράλληλα με το μητρικό θηλασμό έλαβε την μερικώς υδρολυμένη φόρμουλα, λαμβάνοντας υπόψη το οικογενειακό ιστορικό ατοπικής δερματίτιδας (p=0,007; RR: 0,29, 95%-CI: 0,09, 0,93). Η συχνότητα εμφάνισης ατοπικής δερματίτιδας στην ομάδα αποκλειστικού θηλασμού δεν διέφερε από αυτή στην ομάδα που ακολούθησε μεικτό τρόπο σίτισης με τη φόρμουλα άθικτης πρωτεΐνης. Όσον αφορά την αλλεργία στην πρωτεΐνη του αγελαδινού γάλακτος, η

ομάδα του αποκλειστικού θηλασμού έδειξε μια τάση (p=0,086) προς υψηλότερη συχνότητα εμφάνισης σε σύγκριση με την ομάδα που έλαβε την μερικώς υδρολυμένη φόρμουλα, ενώ δεν υπήρχε διαφορά με την ομάδα που έλαβε την φόρμουλα άθικτης πρωτεΐνης. Μεταξύ των δύο ομάδων που σιτίστηκαν με φόρμουλα, δεν υπήρχε διαφορά στη συχνότητα εμφάνισης αλλεργίας στην πρωτεΐνη του αγελαδινού γάλακτος στους 6 μήνες της παρέμβασης.

Συμπεράσματα: Ο συνδυασμός θηλασμού και μερικώς υδρολυμένης φόρμουλας οδήγησε σε χαμηλότερη επίπτωση ατοπικής δερματίτιδας σε έναν υποπληθυσμό των βρεφών της μελέτης με θετικό οικογενειακό ιστορικό για ατοπική δερματίτιδα. Επιπλέον, βρέθηκε μια τάση προς χαμηλότερη συχνότητα εμφάνισης αλλεργίας στην πρωτεΐνη του αγελαδινού γάλακτος στην ομάδα που παράλληλα με το μητρικό θηλασμό έλαβε την μερικώς υδρολυμένη φόρμουλα, σε σύγκριση με την ομάδα του αποκλειστικού θηλασμού. Η ομάδα ομάδα του αποκλειστικού θηλασμού δεν διέφερε από την ομάδα που έλαβε την φόρμουλα άθικτης πρωτεΐνης στις συχνότητες εμφάνισης ατοπικής δερματίτιδας και αλλεργίας στην πρωτεΐνη του αγελαδινού γάλακτος. Η παρούσα μελέτη δείχνει ότι τα βρέφη με αυξημένο κίνδυνο για εμφάνιση αλλεργίας στην πρωτεΐνη του αγελαδινού γάλακτος μπορεί να ωφεληθούν, όσον αφορά την εμφάνιση ατοπικής δερματίτιδας, από έναν συνδυασμό μητρικού γάλακτος και της μερικώς υδρολυμένης φόρμουλα για βρέφη που μελετήθηκε στην παρούσα μελέτη.

Λέξεις κλειδιά: αλλεργία στην πρωτεΐνη του αγελαδινού γάλακτος, ατοπική δερματίτιδα, μερικώς υδρολυμένη φόρμουλα, τρόποι σίτισης, βρεφική φόρμουλα

ABSTRACT

Background: The prevalence of Cow's Milk Protein Allergy (CMPA) has increased in developed countries in the last four decades, especially in children younger than 6 years of age. Moreover, presence of family history of atopy and atopic dermatitis (AD) have been well recognized as risk factors for CMPA, while partially hydrolyzed formulas (pHF) have been suggested as means to prevent the development of allergic manifestations in high-risk infants who are not exclusively breastfed. However, the role of this strategy has recently been questioned based on the lack of robust scientific evidence supporting their efficacy in allergy prevention.

Objectives: The aim of this study is to examine the effect of exclusive breastfeeding with the effects of the mixed feeding (breastmilk combined with pHF or SF) on the development of CMPA, AD and growth parameters in high-risk infants within the first 6 months of life.

Methods: The present study is a multicenter, double-blinded, parallel, randomized controlled study. Participating infants were exclusively breastfed or randomly allocated to one of the two intervention formulas: a) a pHF or b) an intact protein (standard) cow's milk formula (SF). The study was implemented in three countries between 2017-2019. The incidence, the relative risk and their 95% confidence interval (RR, 95% CI) were calculated for the occurrence of CMPA and AD within the first six months of life. Furthermore, a Poisson General Equation Estimation (GEE) regression analysis was performed to calculate the treatment x time interaction effects on the incidence of CMA and AD in the pHF or SF groups compared to the EBF group. The GEE regression analysis was also stratified for the family history of AD.

Results: After 6 months of intervention, the incidence of AD was higher in the EBF infants than in the pHF group, when positive family history of AD was present (p=0.007; RR: 0.29, 95%-CI: 0.09, 0.93). The incidence of AD in the EBF group did not differ from that in the SF group. Regarding CMPA, EBF showed a trend (p=0.086) towards a higher incidence as compared to pHF, whereas there was no difference with the SF group. Between the two formula-fed groups (pHF & SF), there was no difference in the incidence of CMPA at 6 months of intervention.

Conclusions: The combination of breastfeeding and pHF resulted in a lower incidence of AD in a sub-population of infants with a positive family history for AD. Moreover, a trend was found towards a lower incidence of CMPA in the pHF group, as compared to EBF. The EBF group did not differ from SF in the incidences of AD and CMPA. The present study indicates that infants with increased risk for CMPA may benefit, from an allergy point of view, from a combination of breastmilk and the studied pHF infant formula.

Keywords: cow's milk protein allergy, atopic dermatitis, partially hydrolysed formula, feeding practices, infant formula

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ABBREVIATIONS

AAF	Amino Acid–based Formula
AD	Atopic Dermatitis
BMI	Body Mass Index
CMA	Cows' Milk Allergy
CMPA	Cows' Milk Protein Allergy
CoMiSS	Cow's Milk-related Symptom Score
DSMB	Data Safety Monitoring Board
EBF	Exclusive Breastfeeding
eHF	extensively Hydrolyzed infant Formula
ESPGHAN	European Society for Paediatric Gastroenterology Hepatology and Nutrition
FPIES	Food Protein – Induced Enterocolitis Syndrome
GMS	Growth Monitoring Studies
OFC	Oral Food Challenge
pHF	partially Hydrolyzed Formula / partially Hydrolyzed whey-based Formula
SCORAD	Scoring of Atopic Dermatitis Index
SF	Standard Formula
SPT	Skin Prick Test
WHO	World Health Organization

1. INTRODUCTION

1.1 Cow's Milk Protein Allergy (CMPA) and Atopic Dermatitis (AD) definitions and prevalence

Food allergy is an immune-based disease proved to be a serious health concern worldwide. Recent research estimates that food allergy affects 5% of children under the age of 5 years and 4% of teens and adults, with an increasing prevalence noted over the past few decades (1). The immune reaction may be immunoglobulin E (IgE)-mediated, non-IgE mediated, or including both types (mixed) (2).

Cows' milk allergy (CMA) or cow's milk protein allergy (CMPA) is an immune reaction to proteins found in cow's milk (3) and is the most common food allergy in infants and young children younger than 3 years (4), (5).

CMPA is categorized as IgE-mediated, non-IgE mediated, and mixed (IgE combined with non-IgE) (6-12). The estimated CMPA prevalence in developed countries is ranging from 0.5% to 3% during first year of life (13-15), while the same estimation for breast-fed infants is 0.5% (16-17). Research data from the EuroPrevall birth cohort study conducted in nine centers in Europe, estimated a mean adjusted incidence of 0.74% (95% CI 0.56–0.97%) of challenge-diagnosed CMPA in children aged under 2 years; however, variations were noted ranging from 0.00% to 1.29% across countries (18).

CMPA symptoms may vary in both their type and their severity between patients, and they can be either "immediate" or "delayed". Immediate symptoms occur from minutes up to 2 hours after allergen ingestion and are more likely to be IgE-mediated, whereas delayed symptoms occur up to 48 hours or even 1 week following the ingestion and may also involve non–IgE-mediated immune mechanisms. Although the privilege manifestations derive from skin, gastrointestinal and respiratory track, the symptoms might involve different organ systems (2). Table 1 summarizes the main symptoms and signs related to CMPA (2).

Table A - Symptoms and signs related to CMPA			
	Infants and toddlers	Older children	Immediate reaction (within min – 2 h after ingesting CMP)
Digestive	Dysphagia Frequent regurgitation Colic, abdominal pain Vomiting Anorexia, refusal to feed Diarrhea ± intestinal protein or blood loss Constipation ± perianal rash Failure to thrive Occult blood loss Iron-deficiency anemia	Dysphagia Food impaction Regurgitation Dyspepsia Nausea, vomiting Anorexia, early satiety Diarrhea ± intestinal protein or blood loss Constipation Abdominal pain Occult blood loss Iron-deficiency anemia	Vomiting
Respiratory	Runny nose Wheezing Chronic coughing (all unrelated to infections)	Runny nose Wheezing Chronic coughing (all unrelated to infections)	Wheezing or stridor Breathing difficulties
Skin	Urticaria (unrelated to infections, drug intake, or other causes) Atopic eczema Angioedema (swelling of lips or eyelids	Urticaria (unrelated to infections, drug intake, or other causes) Atopic eczema Angioedema (swelling of lips or eyelids	Urticaria Angioedema
General	Anaphylaxis Shock-like symptoms with severe metatobolic acidosis, vomiting, and diarrhea (FPIES)	Anaphylaxis	Anaphylaxis FPIES
CMPA: cow's-milk protein allergy FPIES: food protein – induced enterocolitis syndrome			

Atopic dermatitis (AD) (also known as atopic eczema) is a common inflammatory skin disease characterized by intense itching and recurrent eczematous lesions (19). AD's clinical characteristics have already been described during ancient times (20). Although it most often starts in infancy and affects approximately 20% of children, atopic dermatitis remains one of the most common chronic diseases affecting the 20% of the population in developed countries, having in parallel a substantial impact on quality of life (21-22). For many years, scientists had been recorded AD as the first manifestation of atopy and the initial step in the well-known atopic march that basically leads to asthma and allergic rhinitis (19).

Prevalence of AD during the last decades has been increased worldwide, especially in the past 30 years. In developed countries, it seems to plateau now at 10–20%, whereas it is lower but continues to increase in many developing countries (23), (24). In most cases, the disease manifests during the first year of life (i.e., early onset), but it can commence at any age. (25), (26). Recent European and USA derived data, exhibit that the prevalence of AD among children is approximately 20% (27-28) and the incidence among children in westernized countries is 9.6% (29).

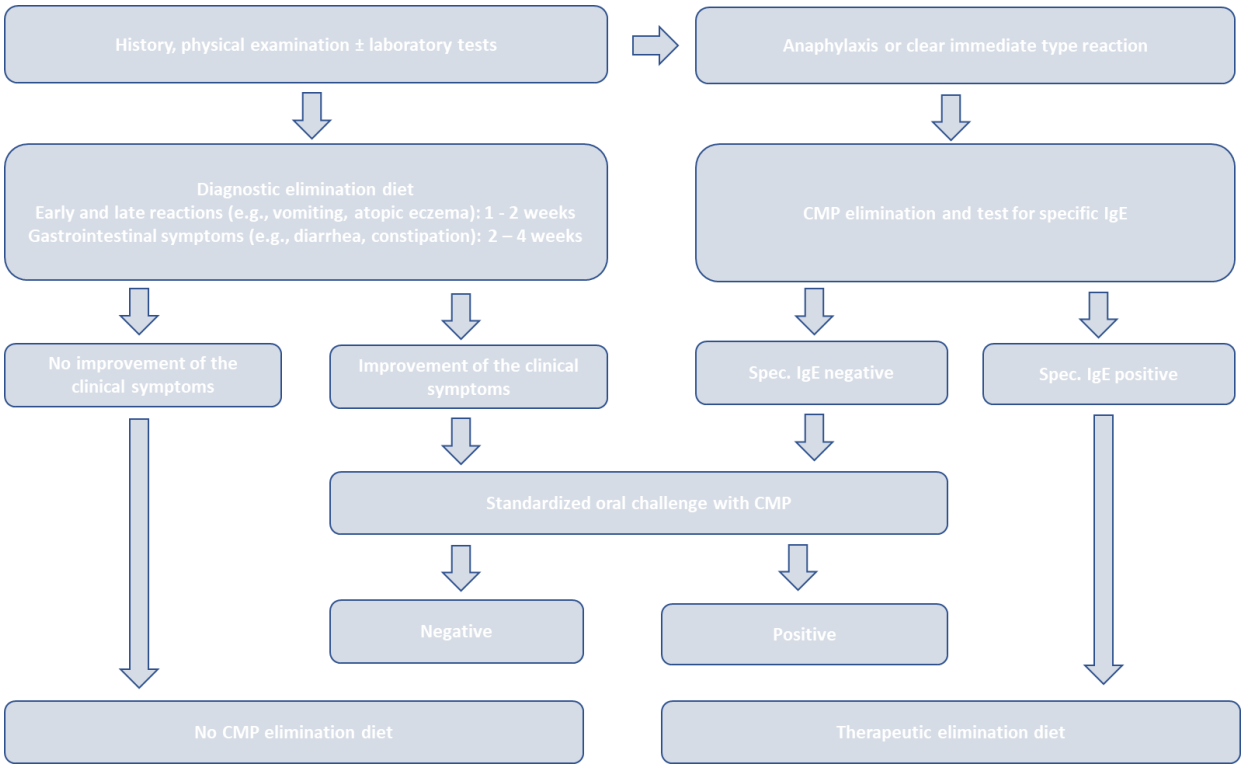
The earliest clinical signs are skin dryness and roughness, but eczematous lesions usually do not occur before the second month of life (19). The clinical symptoms can last for extensive periods or follow a relapsing–remitting pattern with recurrent flare-ups (25), (26). The disease is mild in about 80% of affected children (30). Birth cohort studies (25), (31) have suggested that in up to 70% of cases, the disease greatly improves or resolves until late childhood and that early and severe onset, family history of atopic dermatitis, and early allergen sensitizations are some of the risk factors related with late resolve of the disease. AD has a substantial impact on quality of life (21), (22), while leads to substantial social and financial costs and accounts for the largest global burden of disability owing to skin diseases (32).

1.2 Cow's Milk Protein Allergy (CMPA) and Atopic Dermatitis (AD) assessment methods

The European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) has provided a practical approach for the diagnosis and management of CMPA (2). The first step of diagnostic procedures includes a thorough medical history and physical examination. The diagnostic work up depends on the type of reaction and may require a skin prick test (SPT), the determination of milk specific IgE in blood, and oral food challenge (OFC) test. In cases of non-IgE or unclear history of immediate symptoms, an elimination diet followed by oral food challenges is the gold standard for establishing CMPA diagnosis. Figure 1 below, displays the

ESPAGHAN's proposed algorithm for infants and children with symptoms suggestive of cow's-milk protein allergy (CMPA).

Figure 1:



Koletzko et al. 2012: Algorithm for infants and children with symptoms suggestive of cow's-milk protein allergy (CMPA).

In case of exclusively breast-fed infants, mothers should be encouraged to continue breast-feeding along with a dairy free diet. For infants with history of immediate symptoms, the maternal elimination diet should be maintained for only 3 to 6 days. On the contrary, when delayed symptoms are suspected, the mother should follow elimination diet for up to 14 days. Should there is no improvement, then it is possible that the symptoms are not due to CMPA and the patient should be further evaluated. Should the symptoms improve, then a reintroduction of CMP into the mother's diet could then be performed. In case this challenge resulted positive, the mother may continue breast-feeding along with the maintenance of dairy free diet, and receive calcium supplements and dietetic counseling to ensure that her nutritional needs are fulfilled (33). In addition, proteins other than CMP (e.g., soy, egg) may cause allergic reactions to breast-feeding infants (34). In such cases, an elimination diet that excludes causative foods may be followed by the mother in parallel with breastfeeding, and only if there is a valuable benefit on the well-being of the infant (2).

A therapeutic formula may replace breastfeeding for a sort of period and maximum to 2 weeks, in breast-fed infants with severe symptoms, such as severe atopic eczema or allergic (entero) colitis complicated by growth faltering (34). Even it lacks evidence, many countries commonly propose the use amino acid–based formula (AAF) for diagnostic elimination in these extremely sick exclusively breast-fed infants. This approach is to stabilize the child's condition while the mother expresses breast milk in transition to her CMP-free diet. In cases in which symptoms reoccur despite a strict CMP-free diet in the mother, further elimination of other highly allergenic foods from the mother's diet or weaning from breast milk to a therapeutic formula is recommended (35), (36).

For non-breast-fed infants, cow's-milk–based formula and supplementary foods containing CMP or other unmodified animal milk proteins (e.g., goat's milk, sheep's milk) should be strictly avoided (37), (38). If the first feeds with cow's-milk–based formula in a breast-fed infant cause symptom, the infant should return to exclusive breast-feeding without any elimination in the maternal diet. An elimination diet in formula-fed infants usually starts with an extensively hydrolyzed infant formula (eHF) with proven efficacy in infants with CMPA (37), (39). In infants with extremely severe or life-threatening symptoms, an AAF may be considered as the first choice. Soy protein–based formula may be an option in infants older than 6 months who do not accept the bitter taste of an eHF, or in cases in which the higher cost of an eHF is a limiting factor, provided that the tolerance to soy protein has been established. If there is no improvement within 2 weeks, then an allergic reaction to the remaining peptides in the eHF must be considered, particularly in infants with sensitization against multiple foods (40), (41). In these cases, an AAF should be tried before CMPA is ruled out as cause of the symptoms. Previous concerns that infants with CMPA would react to residual protein traces in lactose have often resulted in complete avoidance of both lactose and CMP. Adverse reactions to lactose in CMPA are not supported in the literature, and complete avoidance of lactose in CMPA is no longer warranted. eHFs containing purified lactose are now available and have been found safe and effective in the treatment of CMPA (42). These formulae may also be more palatable for infants older than 6 months. It is, however, possible for secondary lactose intolerance to coexist in infants who have enteropathy with diarrhea, and therefore a lactose-free eHF will be required initially in these cases.

Regarding the evaluation of CMPA symptoms, the Cow's Milk-related Symptom Score (CoMiSS) has been validated as an awareness tool for the evaluation of non-IgE mediated CMPA

symptoms, based on a combination of general discomfort, gastrointestinal, respiratory and dermatological symptoms (43).

In respect to AD, no specific laboratory or histological findings have been reported, and thus the diagnosis relies exclusively on clinical features. Several sets of diagnostic criteria have been developed, such as the Hanifi and Rajka criteria, and an empirically derived, simplified version distinguishing essential, common, and associated features, which are useful in the clinical setting (44). However, the nine-region Scoring of Atopic Dermatitis Index (SCORAD) has been preferred for assessing disease severity (45).

1.3 Cow's Milk Protein Allergy (CMPA) risk factors

Family history of allergy-associated diseases is the main risk factor for allergy manifestations in children. Primary prevention strategies could be beneficial in infants having a first-degree relative with a history of allergy, also known as high-risk infants (46), (47). Literature supported data shown that children with CMPA are more likely to be boys and are also more likely to have in parallel other atopic diseases, such as more than one food allergy (48), (49), asthma, atopic dermatitis, and allergic rhinitis (50). There is also some evidence from studies suggesting that race/ethnicity may related to CMPA allergy manifestations. Specifically, non-Hispanic black and non-Hispanic white children are more likely to be sensitized to milk, based on serum IgE (51), (52). There is evidence that genetic, epigenetic, and environmental factors play an important role in the development of CMPA, although underlying mechanisms are still being elucidated (53), (54). CMPA has been estimated to be 15% heritable (55), likely due to multiple genetic variants with small effect sizes.

The prenatal and early childhood environment also plays a role, which is demonstrated by estimates of risk in immigrant populations. Among NHANES 2005–2006 participants aged 0 to 21 years, those who were US-born had a greater than 2-fold higher odds of sensitization to milk; among US-born children, those from immigrant families had a 1.7-fold higher risk of sensitization than children from non-immigrant households. There is evidence that children who immigrated in early life have a higher risk of sensitization to cow's milk than those who immigrated later in life. Among immigrant children, those who immigrated prior to age 2 years had non-significantly increased odds of sensitization to cow's milk (OR 3.47, $p = 0.09$) (56). It will be important to further investigate the prenatal and early childhood exposures that underlie these differences and the implications on clinically relevant allergy.

Presence of family history of atopy and AD have been well recognized as risk factors for CMPA, while CMPA affects a significant proportion of infants especially with early and severe AD (19).

1.4 Infant formulas containing hydrolysed protein for prevention of Cow's Milk Protein Allergy (CMPA) and Atopic Dermatitis (AD)

Partially hydrolyzed formula (pHF) generally contains peptides with molecular weights around < 5,000 Da (57). The partial hydrolysis removes part of the sensitizing epitopes which reduces the allergenicity of the proteins and their ability to induce sensitization, while retaining sufficient size of peptides to stimulate the induction of oral tolerance (58), (59). The role of partially hydrolyzed formulae (pHF) for primary prevention of CMA has been long debated (60). The longest, largest, longitudinal studies on pHF, the German Infant Nutritional Intervention Study (GINI study) which started in early 2000 showed that whey pHF was significantly associated with reduction of AD manifestations up to the age of 15 years old (61-65) and reduced the risk for asthma and other respiratory symptoms (66). However, a more recent study with pHF enriched with pre-biotic oligosaccharides did not show any preventive effect (67).

Furthermore, there is no consensus with regards to early exposure to intact CMP in the first few weeks of life and the risk of CMA in later life. A large observational cohort study showed that delayed introduction of cow's milk products in the infants' diet was associated with an increased risk of developing atopy at 2 years of age, especially AD (68). A prospective study assessing the risk factors for CMA found early (within 14 days of life) introduction of CMP formulae presented lower rates of Ig-E mediated CMA compared to those with late consumption at 105- 194 days of life [0.05% versus 1.75% respectively ($p < 0.001$)] (69). A recent study showed that early continuous exposure to CMP may reduce the risk of CMA; while an introduction to CMP formulae during the first 3 days of life followed by complete CMP avoidance until the child is weaned may have the opposite effect (70).

In view of prevalence, comorbidities and cost for the healthcare system primary prevention strategies have been developed in respect to feeding practices (71), (72), (73), (74). Exclusive breastfeeding is recommended for at least 4 months of age for its major benefits on infant health by transferring immune factors and protection against infections; nevertheless, the potential protective effect on allergic disease occurrence remains controversial (75), (76), (77), (78). Moreover, strategies for primary prevention of food allergy CMPA are still inconclusive. Introduction of partially hydrolyzed formulas (pHF) in high-risk infants has been considered to reduce the risk of CMPA symptoms; however, this strategy has been currently challenged (79), (80), (81). Noteworthy, early (after the 1-4 postnatal weeks) and daily supplementation of small amounts of intact protein cow's milk formula to breastfed infants (mixed feeding) has recently

been shown to prevent milk sensitization and CMPA (82), (83), (84). Of interest, mixed feeding, in the first three months of life has been shown to decrease CMPA manifestations (85). Recent research has reported that a specific partially hydrolyzed whey-based formula (pHF) reduced the risk of AD development, particularly in those with a family history of AD, and tended to reduce the development of CMPA in non-exclusively breastfed infants at high-risk for allergy, as compared to a formula with intact protein (86).

Within this concept, partially hydrolyzed formulas (pHF) have been suggested as means to prevent the development of allergic manifestations in high-risk infants who are not exclusively breastfed (6, 7). Several studies including meta-analyses and systematic reviews were indicative of a risk-reducing effect of pHF particularly for AD (8–12). However, the role of pHF has recently been questioned based on the lack of robust scientific evidence supporting their efficacy in allergy prevention (13, 14). Beyond differences in study designs, hydrolysates used and methodological limitations, concerns for conflict of interest and publication bias have also been raised, as research in this field is usually sponsored by milk formula manufacturers (7, 14).

According to the U.S. Food and Drug Administration and European legislation, growth monitoring studies (GMS) are necessary to show safety of formulas with new protein fractions, during the period when formula is the sole source of nutrition. Several studies assessing weight gain of breastfed infants as compared to SF-fed infants showed differences in growth velocity between the 4-10th week of life, potentially reflecting differences in protein concentrations (87), (88). Formulas with extensively hydrolyzed milk protein (eHF) have shown less accelerated growth as compared to SF (89). A pHF with low protein (1.9 g/100 kcal) showed a higher weight gain in 4 months' time compared to eHF (2.3 g/100 kcal) (90). With regards to effects on growth in mixed fed (either pHF or SF with breastmilk) infants, no differences (noninferiority) have been reported, while mixed feeding with pHF closely tracked EBF (91). No differences were also reported for fully pHF and SF groups, whereas both formula groups showed a higher weight at 4 months as compared to breastfed infants (92).

1.5 Research question

In the present study, the effect of exclusive breastfeeding was compared with the effects of the mixed feeding groups (breastmilk combined with pHF or SF formula) on the development of CMPA, AD and growth parameters in high-risk infants within the first 6 months of life.

2. METHODOLOGY

2.1 Study design and participants

The Allergy Reduction Trial (A.R.T.) is a multicenter, double-blinded, parallel, randomized controlled study assessing differences in the incidence of Cow's Milk Protein Allergy (CMPA) and atopic dermatitis (AD) within the first six months of life in apparently healthy term infants at high risk of developing allergy (family history of allergy). Two intervention formulas were provided to study infants, fed exclusively or as supplementary to breastfeeding: a) a partially hydrolyzed whey-based formula (Frisolac Gold preventive HA) or b) an intact protein cow's milk formula (Frisolac Gold; standard formula; SF). Exclusively breastfed infants were also followed as a parallel observational group. The study was conducted in 6 centers in 3 countries: Bulgaria (1), Cyprus (1) and Greece (4) between 2017-2019.

Due to increased dropout and reduced recruitment rate during the first months of the study, the initial protocol was amended to include a lower number of follow-up visits, additional recruiting centers, recruitment-period time extension and sample size re-estimation.

The study protocol, information letter to the parents/legal guardians, and written informed consent form were reviewed and approved by the appropriate independent ethics committee in each center: Bulgaria: Research Ethics Committee of Medical University of Varna; Cyprus: Cyprus National Bioethics Committee; Greece: a) Research Ethics Committee of Alexandra Hospital, b) Research Ethics Committee of Aretaieio Hospital, c) Research Ethics Committee of Attikon Hospital, d) Research Ethics Committee of Helena Hospital. The study was conducted in accordance with the guidelines of the Declaration of Helsinki, the International Conference on Harmonization (ICH) guidelines on Good Clinical Practice (GCP) and was registered in the Netherlands Trial Registry [Identifier: Trial NL6120 (NTR6259)].

2.2 Recruitment procedures

During the 7^{th} – 9^{th} month of gestation (or shortly after delivery), families that were attending public and private maternity clinics, were interviewed by study researchers regarding family history of allergy, using an enriched validated questionnaire (93) to identify infants at high-risk of developing allergy. Family history of allergy included Atopic Dermatitis/Eczema, Allergic Asthma, Allergic Rhinitis-Conjunctivitis, Urticarial rash with exposure to pets and Food Allergy in parents and/or siblings. Only cases with a doctor diagnosis of allergic disease in the family were

considered to be eligible. Parents were then informed about the A.R.T. study and depending on their willingness to participate, a pre-consent form was requested to be signed. On the day of delivery parents were given the Parent Information Brochure which included detailed information regarding the study procedures. Within four days after delivery, if the inclusion criteria were fulfilled and parents had decided to participate in the study, a consent form was completed.

2.3 Inclusion Criteria

Only term infants (≥37weeks), with birth weight ≥2500g, postnatal age <5 days, apparently healthy with no signs of allergy having at least one parent or sibling with confirmed by a doctor history of allergy, who were exclusively breastfed or fed with an extensively hydrolyzed infant formula (eHF) since birth could be recruited. The parents should be willing to bring their infants to the study centers for at least bi-monthly follow-up visits during the first six months (and to answer a telephone questionnaire at the age of 12 months).

2.4 Randomization and Treatment Allocation

All mothers were strongly encouraged to breastfeed. However, parents could choose freely the type of their infant's feeding (exclusive breastfeeding, mixed-feeding or exclusive formula-feeding). In case of non-exclusive breastfeeding, subjects were at random allocated to the pHF or the SF as soon as consent was obtained by the parents. An independent statistician created computer-generated randomization tables for each study center. Stratification for gender, type of feeding (exclusively formula-fed or mixed-fed) and presence of AD in a parent and/or sibling was applied.

In case a subject was initially exclusively breastfed, and breastfeeding was insufficient, or the parents wished to add a complementary infant formula before the age of 10 weeks, then the infant could be allocated to one of the two formula groups.

2.5 Study Products, Blinding and Adverse Events

The intervention formulas consisted of a partially hydrolyzed whey-based formula (pHF; Frisolac Gold preventive HA) and an intact protein/standard formula (SF; Frisolac Gold) nutritionally suitable for the first six months of life. Table A (Appendix) presents the macronutrients distribution of the two study products. Both are commercially available and are produced in the Netherlands by FrieslandCampina. The formulas were packed in identical white unlabeled 400g tins carrying the description "not for commercial use" and were distinguished by a different

code-name (FCA or FCB) printed at the bottom of the tins in a small font-size. This was performed by personnel of the packaging department at FrieslandCampina that were not involved in the study. All study personnel and parents were blinded to the study formulas code-matching groups until the whole study was completed and database was locked. Study products were provided for free to the study participants during the first six months of life.

All adverse events (including those classified as serious) and actions taken to resolve them were recorded throughout the study. An independent Data Safety Monitoring Board (DSMB; 2 persons including a Dutch pediatrician and a Dutch allergist) evaluated and discussed the accumulated adverse events at least twice a year to monitor participant safety and make recommendations concerning the continuation, modification or termination of the trial. Two sealed code-break envelopes (each one corresponding to the respective study formula codes FCA and FCB) were kept securely locked and upon request, the specific envelope would have been made available for de-blinding. Still, no "code-break" requests occurred throughout the study and de-blinding did not take place.

2.6 Follow-up evaluation and compliance

Ten days after delivery, the research team contacted the parents by phone to assess the protocol compliance (no formula allowed except the study formulas). Infants visited the study centers for follow-up assessments bi-monthly (at 2nd, 4th, and 6th month) during the first six months of life. Optional follow-ups were performed at the age of one and three months and additional visits were performed at any time point if needed (development of any signs of allergy or adverse events). Infants had not consumed any other formula prior to allocation and solid foods were allowed only after the age of 4 months. No dietary restrictions were advised to breastfeeding mothers.

Formula intake compliance was evaluated using a 7-day milk diary completed during the week preceding the 1st, 2nd, 4th, and 6th month of age. At 1 month of age, milk intake was reported via phone calls (or during an optional follow up visit). If formula consumption was less than 40 mL/kg body weight/day during the 1st month of life or less than 60 mL/kg body weight/day during the 2nd month (and thereafter), then the infant was considered as a dropout. These cut-off values assured a formula intake of about 25% and 40%, respectively, of total daily milk intake (94). For infants that were initially exclusively breastfed and allocated to a study formula before the age of 10 weeks, the same formula quantity requirements were applied.

At the follow-up visits, infants were clinically examined by experienced pediatricians and nurses for the presence of CMPA and AD. Suggestive CMPA and AD symptoms were objectively scored across centers using the Scoring for Atopic Dermatitis (SCORAD) (45) tool and the awareness Cow's Milk-related Symptom Score (CoMiSS) tool (95), (96). SCORAD comprises skin symptoms (location, extend and severity of erythema, oedema, oozing, excoriations lichenifications, dryness and subjective symptoms), and CoMiSS comprises crying, regurgitations, stools, skin and respiratory symptoms. Furthermore, the "Screening for IgE- and non-IgE-mediated food allergy symptoms questionnaire" was completed (97). CMPA was confirmed by oral food challenge by a pediatrician or a pediatric allergist. When an infant was diagnosed with CMPA, management according to the individual national health care system in each country was applied, ensuring that the affected subject would receive the appropriate hypoallergenic formula.

Anthropometric measurements focused on weight, length and head circumference were performed by the same two well-trained research team members at each center, using calibrated digital infant scales (SECA 104 354) with a precision of +/- 20g for weights below 20Kg, an infantometer (SECA 210) measuring to the nearest 0.1cm and a non-elastic tape (SECA 211) measuring to the nearest 0.1cm, respectively. All measurements were performed in triplicates and averaged. In case a pair-wise difference between the three measures was >100g for weight, >0.7cm for length and >0.5cm for head circumference then, a 4th measurement was performed, and the three nearest measurements were written averaged.

2.7 Definition of study Outcomes

Two primary outcomes were defined: CMPA and AD.

Cow's Milk Protein Allergy

CMPA in formula-fed infants was defined as presence of AD (as below) and/or allergic urticarial rash and/or gastro-intestinal manifestations combined with positive oral food challenge (OFC) or suspected CMPA based on clinical evaluation.

CMPA in exclusively breast-fed infants, was confirmed by cow's milk protein (CMP) elimination in the breastfeeding mother's diet for 7-14 days (depending on timing of symptoms disappearance) followed by CMP reintroduction in maternal diet. If symptoms reappeared, then the infant was diagnosed with CMPA.

Atopic Dermatitis

AD was defined as the clinical diagnosis by the pediatrician (typical morphology and distribution of skin lesions, head, neck, trunk and extensor surface of the extremities) and extend and severity were objectified with the recorded SCORAD (total objective score > 1) in combination with the supportive awareness tool CoMiSS (score for Skin Symptoms on Atopic Eczema > 1).

Growth parameters

Body weight, length, and head circumference during the first six months of life, as described earlier. Z-scores for weight, length and BMI were calculated and compared with World Health Organization (WHO) growth charts (https://www.who.int/tools/child-growth-standards/standards).

2.8 Reasons for dropping-out (early termination)

Infants were considered as dropouts if a) parents withdrew consent (and they were allowed to do so at any time-point of the study if they wished), b) infant was fed with any other than the allocated study formula (or an eHF/AAF), c) an exclusively breastfed infant switched to mixed-feeding after the age of 10 weeks, d) formula intake during the 1st and 2nd months of life was less than 40 and 60mL/KgBW/d respectively, e) weaning foods were introduced before the age of 4 months, and f) infant had experienced a Severe Adverse Event.

2.9 Sample size estimation

The sample size calculation was based on two studies: the GINI study of Von Berg et al. (2003) (93), and a study performed by Halken et al. (2000) (98), called 'the Halken study'. Furthermore, the practical limitation that all high-risk infants had to be recruited within 1 to 1.5 years was taken into account. Working with the data from GINI and Halken, the anticipated incidence in the SF group was estimated at 16% vs. 5% (4.7% rounded) in the pHF group, and thus a protective effect size of 68% was anticipated. Using a significance error of 5% (2-tailed) and power of 80%, a sample size of 121 infants per treatment arm should be available for evaluation. Assuming a drop-out rate of 30%, 158 infants had to be included per treatment arm.

2.10 Statistical analysis

All statistical analyses were conducted using the SPSS statistical software for Windows (IBM, version 28.0; IBM, Armonk, NY, USA). The normality of the distribution of continuous variables

was tested by the Kolmogorov–Smirnov test. Normally distributed continuous variables are presented as means and standard deviations (SD), while non-normally distributed ones are presented as medians and interquartile ranges (IQR). Categorical variables are presented as percentages (%).

Both per protocol (PP) and intention–to-treat (ITT) statistical analyses were performed. For the current thesis, ITT analysis was utilized. Between-group differences of continuous variables were tested using either one-way Analysis of Variance (ANOVA) or the non-parametric Kruskal-Wallis test for normally and non-normally distributed variables, respectively. The significance of the association between categorical variables was examined using the chi-squared (χ^2) test or the Fisher exact test, whenever appropriate.

The incidence, the relative risk and their 95% confidence interval (RR, 95% CI) were calculated for the occurrence of AD, Allergic Manifestations (AM) and CMPA within the first six months of life. Furthermore, a Poisson General Equation Estimation (GEE) regression analysis was performed to calculate the treatment x time interaction effects on the incidence of AD, AM and CMA in the pHF or SF groups compared to the EBF group (Model 1). The GEE regression analysis was also stratified for the family history of AD (Model 2). In both GEE regression models, adjustments were also made for the same “confounding factors” that were used in the Freedman test for repeated measures.

Repeated measures ANOVA was used to examine the between-group differences (treatment effect) in infants’ growth indices (i.e., body weight. Length, BMI, and their z-scores) at baseline, 4 and 6 months of age, the within-group changes (time effect) from baseline to the follow-up time-points in each treatment arm, and the differences among treatment arms in the changes from baseline to the 6-month follow-up (treatment x time interaction effect). Adjustments were made for the potential “confounding effect” = of gender, infant’s birth weight, maternal and paternal educational level, region of residence (i.e., urban vs rural) and the country of infant’s birth.

All reported P values were two-tailed, and the level of statistical significance was set at $p< 0.05$.

3. RESULTS

Of 650 subjects eligible for participating in the study, 99 were excluded before being assigned to any group, 331 infants were randomized to one of the two study formula groups and 220 were exclusively breastfed. The flow of the study population and reasons for dropouts are presented in Figure 2.

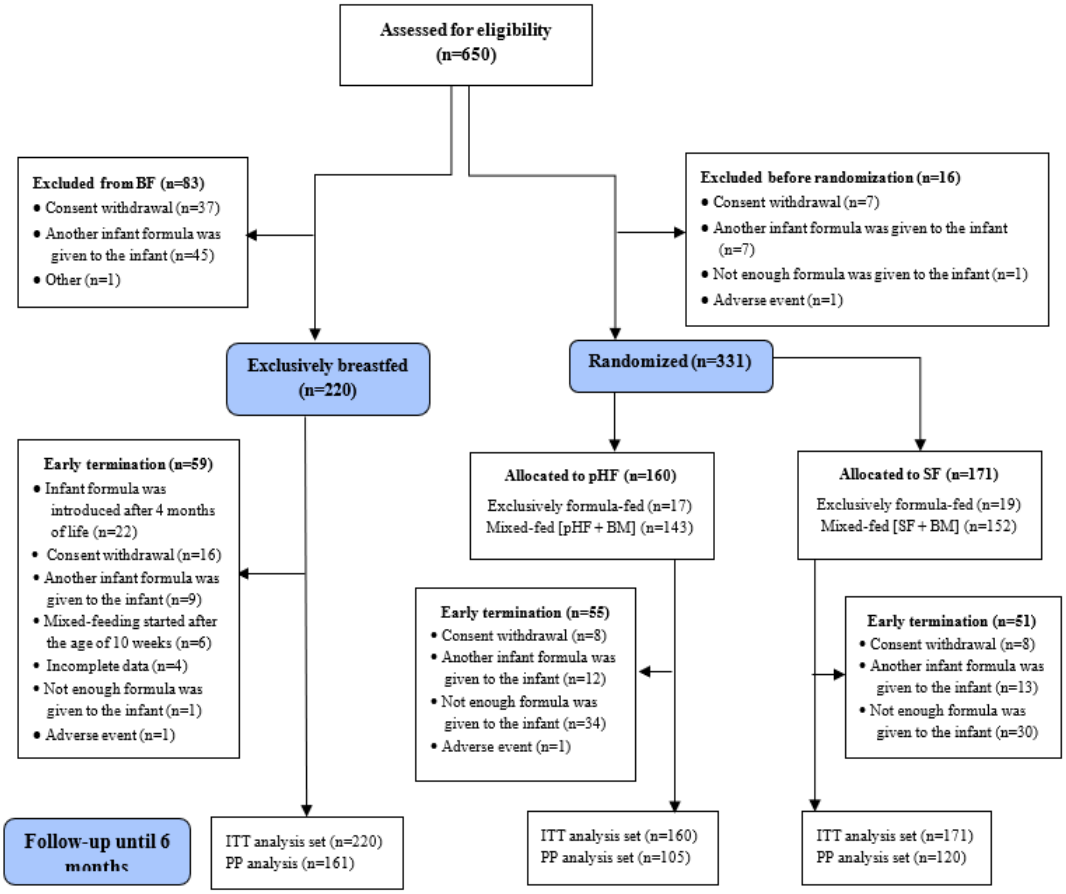


Figure 2: Flow diagram of the A.R.T. population

3.1 Study populations at baseline

Table 1 shows that the majority (n=114 out of n=220; 51.8%) of EBF infants was recruited in Greece (p<0.001), whereas formula fed or mixed fed infants in particular came from Cyprus and Bulgaria (n=274 out of n=331; 82.8%) (p<0.001). Within the countries, Greece also recruited more EBF infants as compared to formula or mixed fed infants. In Bulgaria this was the other way around. In Cyprus the infants were equally distributed over the study groups. Furthermore, relative differences in normal conception and gestational age between the study groups are small and most likely not relevant. Less caesarean deliveries were seen in the group of EBF (p<0.001), as compared to pHF and SF groups.

Regarding anthropometry, at baseline head circumference was different between the study groups (p=0.011), being smallest in the SF group. This group also showed, as compared to EBF, more smoking mothers and fathers at baseline (p<0.038), a lower educational level of both father and mother (p<0.027), and a lower contribution of urban families (p=0.008). At baseline, all infants had negative z-scores for weight and body mass index, with EBF showing the best z-scores for weight as compared to pHF and SF, and for the BMI z-score as compared to SF. For length only the pHF group showed a negative z-score, but groups were not different from each other (Table 3).

3.2 Effect of intervention

After 6 months of intervention (Table 2), the incidence of AD was higher in the EBF infants than in the pHF group, at least when AD was present (p=0.007; RR: 0.29, 95%-CI: 0.09, 0.93) in the family history of allergy. The incidence of AD in the EBF group did not differ from that in the SF group. Regarding CMPA, EBF showed a trend (p=0.086) towards a higher incidence as compared to pHF, whereas there was no difference with the SF group. Between the two formula-fed groups (pHF & SF), there was no difference in the incidence of CMPA at 6 months of intervention. (Table 4).

Absolute body weight, length, head circumference and BMI increased in all groups during the study, with the SF group showing the significantly higher increases in body weight and BMI from baseline to the age of 4 and 6 months, while length changes were more pronounced in the pHF group (Table 3).

Z-scores for length in the formula groups improved during the study, being significant for pHF at 4 and 6 months, and for SF at 6 months. Improvement of the length z-score in the EBF group was not significant, and lower as compared to the formula groups (Table 3). For weight, the z-scores initially decreased in EBF (significant) and pHF (not significant) as shown at age 4 months. Z-scores in both groups increased thereafter. For the SF group, the z-score for weight improved from baseline onwards and was well above the z-scores of the pHF and EBF groups which were not different from each other (Table 3). In line with this, the z-score for BMI was different between the groups at age 4 and 6 months, with SF showing the highest z-scores.

Table 1. Descriptive characteristics of study participants allocated to the three treatment arms at baseline					
	EBF group (N=220)	**pHF group (N=160)**	**SF group (N=171)**	**p-value**	**Total sample (N=551)**
Infant's characteristics					
Country of infant's birth					
Bulgaria, n (%)	48 (31.8)[a,b]	76 (47.5)[a]	82 (48.0)[b]	**<0.001**	206 (37.4)
Cyprus, n (%)	58 (26.4)	55 (34.4)	61 (35.7)		174 (31.6)
Greece, n (%)	114 (51.8)[a.b]	29 (18.1)[a]	28 (16.4)[b]		171 (31.0)
Normal conception, n (%)	215 (98.2)[a]	148 (93.1)[a]	164 (95.9)	**0.045**	527 (96.0)
Gestational age, weeks, mean (SD)	38.9 (1.0)[a]	38.6 (1.2)[a]	38.7 (1.0)	**0.025**	38.8 (1.1)
Caesarean delivery, n (%)	101 (45.9)[a,b]	106 (66.3)[a]	106 (62.0)[b]	**<0.001**	313 (56.8)
Birth weight, g, mean (SD)	3303.6 (392.3)	3270.5 (433.6)	3278.1 (453.7)	0.722	3286.1 (423.6)
Weight at baseline, g, mean (SD)	3258.4 (395.7)	3207.9 (415.8)	3204.2 (439.9)	0.349	3226.9 (415.7)
Length at baseline, cm, mean (SD)	50.1 (2.0)	49.7 (1.8)	49.8 (1.8)	0.075	49.9 (1.9)
Head circumference, cm, mean (SD)	34.4 (1.2)	34.2 (1.2)	34.1 (1.1)	**0.011**	34.2 (1.2)
Gender, female, n (%)	104 (47.3)	67 (41.9)	78 (45.6)	0.575	249 (45.2)
Mother's characteristics					
Age, years, mean (SD)	32.5 (4.9)	31.7 (5.1)	31.3 (5.1)	0.062	31.9 (5.1)
Educational level					
≤ 14 years, n (%)	60 (27.3)[b]	60 (37.5)	70 (40.9)[b]	**0.012**	190 (34.5)
>14 years, n (%)	160 (72.7)[b]	100 (62.5)	101 (59.1)[b]		361 (65.5)
Mother smoking during pregnancy, n (%)	13 (5.9)	15 (9.4)	22 (12.9)	0.059	50 (9.1)
Mother smoking at baseline, n (%)	25 (11.4)[b]	28 (17.5)	41 (24.0)[b]	**0.004**	94 (17.1)
Father's characteristics					
Age, years, mean (SD)	35.1 (5.6)	34.5 (5.7)	34.1 (5.6)	0.210	34.6 (5.6)
Educational level					

≤ 14 years	92 (41.8)[b]	76 (47.8)	95 (55.6)[b]	**0.026**	263 (47.8)
>14 years	128 (58.2)[b]	83 (52.2)	76 (44.4)[b]		287 (52.2)
Father smoking at baseline, n (%)	82 (37.3)[b]	77 (48.1)	83 (48.5)[b]	**0.037**	242 (43.9)
Family characteristics					
Family members at home, median, IQR	4.0 (1.0)	3.0 (1.0)	3.0 (1.0)	0.329	3.0 (1.0)
Urban residence, n (%)	203 (92.3)[b]	146 (91.3)	141 (82.9)[b]	**0.008**	490 (89.1)
Presence of pets indoors at home, n (%)	39 (17.7)	32 (20.0)	38 (22.2)	0.207	109 (19.8)
Medical history					
Family history of:					
Allergic asthma, n (%)	66 (30.0)	42 (26.3)	42 (24.6)	0.462	150 (27.2)
Rhinitis, n (%)	130 (59.1)	81 (50.6)	85 (49.7)	0.118	296 (53.7)
Atopic dermatitis, n (%)	75 (34.1)	46 (28.7)	44 (25.7)	0.186	165 (29.9)
Urticaria, n (%)	32 (14.6)	22 (13.8)	28 (16.4)	0.789	82 (14.9)
Food allergy, n (%)	70 (31.8)	45 (28.1)	52 (30.4)	0.741	167 (30.3)
Occurrence of early life infections in infants					
No infections, n (%)	176 (80.0)	114 (71.3)	127 (74.3)	0.177	417 (75.7)
Before 1st month, n (%)	7 (3.2)	3 (1.9)	6. (3.5)		16 (2.9)
After 1st month, n (%)	37 (16.8)	43 (26.9)	38 (22.2)		118 (21.4)

EBF: exclusive breastfeeding; **pHF:** partially hydrolysed formula; **SF:** Standard formula; **PP:** Per-Protocol; **N:** Number of study participants; **n:** number of non-missing observations; **SD:** Standard Deviation; **IQR:** Interquartile Range.

p-values for the comparison of categorical variables derived from the chi-square test or the Fisher exact test, whenever appropriate. P-values for the comparison of continuous variables derived from one-way ANOVA or the Kruskal Wallis test for normally and non-normally distributed variables respectively. All p-values in bold indicate statistically significant differences among treatment arms. Percentages sharing the same superscript letter within the same raw are statistically significantly different between them, according to pairwise comparisons using the Bonferroni correction to account for type I error.

Table 2. Incidence and relative risk for CMPA, AD and AM within the first six months of life in exclusively breastfed, exclusively formula-fed and mixed-fed infants								
	Treatment Arms			**RR_1 (95% CI) (PHF/ExcBF)**	**p-value$_1$**	**RR_2 (95% CI) (SF/ExcBF)**	**p-value$_2$**	**p-value$_3$**
	EBF	**pHF**	**SF**					
Model 1	**(N=220)**	**(N=160)**	**(N=171)**					
AD, n (%)	38 (17.3)	17 (10.6)	32 (18.7)	0.62 (0.36, 1.05)	0.064	1.08 (0.71, 1.66)	0.709	0.069
CMPA, n (%)	21 (9.5)	8 (5.0)	16 (9.4)	0.52 (0.24, 1.15)	0.086	0.98 (0.53, 1.82)	0.953	0.154
Model 2								
FHAD (+)	**(N=75)**	**(N=46)**	**(N=44)**					
AD, n (%)	**17 (22.7)**	**3 (6.5)**	12 (27.3)	**0.29 (0.09, 0.93)**	**0.007**	1.20 (0.63, 2.28)	0.576	**0.003**
CMPA, n (%)	12 (16.0)	3 (6.5)	7 (15.9)	0.41 (0.12, 1.37)	0.088	0.99 (0.42, 2.34)	0.990	0.166
FHAD (-)	**(N=145)**	**(N=114)**	**(N=127)**					
AD, n (%)	21 (14.5)	14 (12.3)	20 (15.7)	0.85 (0.45, 1.59)	0.628	1.08 (0.62, 1.91)	0.769	0.751
CMPA, n (%)	9 (6.2)	5 (4.4)	9 (7.1)	0.71 (0.24, 2.05)	0.525	1.14 (0.47, 2.79)	0.770	0.651

AD: atopic dermatitis; **CMPA:** cow's milk protein allergy, confirmed by oral food challenge; **EBF:** exclusive breastfeeding; **pHF:** partially hydrolysed formula; **SF:** standard formula; **N:** number of study participants; **RR_1:** Relative risk for CMPA or AD in Exc BF vs pHF; **RR_2:** Relative risk for CMPA or AD in Exc BF vs SF; **CI:** Confidence Interval; **FHAD (+):** family history of AD; **FHAD (-):** no family history of AD;

Model 1 was adjusted for the potential confounding effect of gender, type of conception (i.e., normal vs IVF), gestational age, type of delivery (i.e., labor vs. caesarean), the amount of human milk consumed by infants, the occurrence of early life infections, maternal and paternal educational level, maternal and paternal smoking at home, the presence of pets at home, the region of residence (i.e., urban vs rural) and the country of infant's birth. Model 2 was further adjusted for the interaction between treatment arm and FHAD.

All p-values derived from Poisson Generalized Estimating Equation (GEE) regression analysis. p-value$_1$ provides the treatment effect in the pHF compared to the EBF group; p-value$_2$ provides the treatment effect in the SF compared to the EBF group; p-value$_3$ indicates the overall treatment x time effect.

Table 3. Changes in infants' growth indices from baseline to 4 and 6 months of age per treatment arm					
	Time-point of evaluation			**Time effect (4-month change)**	**Time effect (6-month change)**
	Baseline	**Visit 2 (4 months)**	**Visit 3 (6 months)**		
	Mean (SEM)	**Mean (SEM)**	**Mean (SEM)**	**Mean change (95% CI)**	**Mean change (95% CI)**
Body weight (kg)					
EBF group (n=219)	3.25 (0.007)[a,b]	6.62 (0.046)	7.65 (0.060)	**3.37 (3.28; 3.46)**	**4.41 (4.29; 4.53)**
pHF group (n=159)	3.22 (0.008)[a]	6.63 (0.052)	7.68 (0.069)	**3.41 (3.31; 3.51)**	**4.47 (4.33; 4.60)**
SF group (n=170)	3.20 (0.008)[b]	6.76 (0.052)	7.82 (0.068)	**3.55 (3.45; 3.66)**	**4.62 (4.49; 4.75)**
Treatment effect (P-value)*	**<0.001**	0.085	0.148	**0.025**	**0.017**
Length (cm)					
EBF group (n=219)	49.9 (0.09)	63.4 (0.17)	67.4 (0.19)	**13.4 (13.1; 13.7)**	**17.5 (17.1; 17.8)**
pHF group (n=159)	49.8 (0.11)	63.6 (0.19)	67.9 (0.21)	**13.8 (13.4; 14.1)**	**18.1 (17.7; 18.5)**
SF group (n=170)	50.0 (0.10)	63.4 (0.19)	67.9 (0.21)	**13.5 (13.1; 13.8)**	**17.9 (17.6; 18.3)**
Between-group effect (P-value)*	0.412	0.770	0.127	0.305	0.056
Body Mass Index (Kg/m^2)					
EBF group (n=219)	13.0 (0.05)[b]	16.5 (0.11)	16.8 (0.12)	**3.5 (3.2; 3.7)**	**3.8 (3.6; 4.1)**
pHF group (n=159)	12.9 (0.06)	16.4 (0.13)	16.7 (0.14)	**3.5 (3.2; 3.7)**	**3.7 (3.5; 4.0)**
SF group (n=170)	12.8 (0.06)[b]	16.8 (0.13)	16.9 (0.14)	**4.0 (3.7; 4.3)**	**4.2 (3.9; 4.4)**
Between-group effect (P-value)*	**0.035**	0.075	0.405	**0.004**	**0.004**
Head Circumference (cm)					
EBF group (n=219)	34.4 (0.07)[b]	41,3 (0.08)	43.0 (0.10)	**6.9 (6.8; 7.1)**	**8.6 (8.5; 8.8)**
pHF group (n=159)	34.2 (0.07)	41.3 (0.10)	42.9 (0.11)	**7.1 (6.9; 7.3)**	**8.7 (8.5; 8.9)**
SF group (n=170)	34.1 (0.07)[b]	41.3 (0.09)	43.0 (0.11)	**7.2 (7.0; 7.4)**	**8.9 (8.7; 9.1)**
Between-group effect (P-value)*	**0.020**	0.997	0.907	0.083	0.057
Weight-for-age z-score					
EBF group (n=219)	-0.13 (0.02)[a,b]	-0.28 (0.06)	-0.13 (0.07)	**-0.15 (-0.27; -0.03)**	0.00 (-0.14; 0.14)
pHF group (n=159)	-0.19 (0.02)[a]	-0.25 (0.07)	-0.08 (0.08)	-0.06 (-0.19; 0.08)	0.12 (-0.04; 0.27)

SF group (n=170)	-0.22 (0.02)[b]	-0.08 (0.07)	0.09 (0.08)	**0.14 (0.009; 0.27)**	**0.31 (0.16; 0.46)**
Treatment effect (P-value)*	**<0.001**	0.069	0.109	**0.006**	**0.003**
Length-for-age-z-score					
EBF group (n=219)	0.04 (0.05)	0.04 (0.08)	0.19 (0.08)	0.002 (-0.14; 0.15)	0.15 (-0.01; 0.31)
pHF group (n=159)	-0.06 (0.06)	0.14 (0.09)	0.43 (0.10)	**0.19 (0.03; 0.36)**	**0.48 (0.30; 0.67)**
SF group (n=170)	0.03 (0.06)	0.08 (0.09)	0.44 (0.09)	0.04 (-0.12; 0.21)	**0.41 (0.23; 0.59)**
Between-group effect (P-value)*	0.419	0.722	0.081	0.206	**0.019**
Body Mass Index-for age z-score					
EBF group (n=219)	-0.34 (0.04)[b]	-0.40 (0.04)	-0.31 (0.09)	-0.06 (-0.23; 0.10)	0.03 (-0.15; 0.21)
pHF group (n=159)	-0.37 (0.05)	-0.43 (0.09)	-0.42 (0.10)[c]	-0.06 (-0.25; 0.13)	-0.05 (-0.26; 0.15)
SF group (n=170)	-0.50 (0.05)[b]	-0.16 (0.09)	-0.22 (0.10)[c]	**0.34 (0.15; 0.52)**	**0.29 (0.09; 0.49)**
Between-group effect (P-value)*	**0.032**	0.069	0.333	**0.003**	**0.002**

EBF: exclusive breastfeeding; **pHF:** partially hydrolysed formula; **SF:** standard formula. All p-values derived from the Analysis of Variance for Repeated Measures. All p-values in bold indicate statistically significant between-group differences among treatment arms, while mean changes in bold indicate within-group changes from baseline to 6 months. Mean values sharing the same superscript letter indicate significant differences between treatment arms in the relevant pairwise comparisons. All statistical analyses were adjusted for the potential confounding effect of gender, infant's birth weight, maternal and paternal educational level, region of residence (i.e., urban vs rural) and the country of infant's birth.

Table 4. Incidence and relative risk for CMPA within the first six months of life in exclusively formula fed and mixed fed infants				
	Treatment Arms		**RR (95% CI) (PHF/SF)**	**p-value**
	pHF	**SF**		
Model 1	**(N=160)**	**(N=171)**		
CMPA, n (%)	8 (5.0)	16 (9.4)	0.53 (0.23, 1.21)	0.124
Model 2				
FHAD (+)	**(N=46)**	**(N=44)**		
CMPA, n (%)	3 (6.5)	7 (15.9)	0.40 (0.11, 1.46)	0.149
FHAD (-)	**(N=114)**	**(N=127)**		
CMPA, n (%)	5 (4.4)	9 (7.1)	0.62 (0.21, 1.79)	0.373

CMPA: cow's milk protein allergy, confirmed by oral food challenge; **pHF:** partially hydrolysed formula; **SF:** standard formula; **N:** number of study participants; **RR:** Relative risk for CMPA pHF vs SF; **CI:** Confidence Interval; **FHAD (+):** family history of AD; **FHAD (-):** no family history of AD.

Model 1 was adjusted for the potential confounding effect of gender, type of conception (i.e., normal vs IVF), gestational age, type of delivery (i.e., labor vs. caesarean), the amount of human milk consumed by infants, the occurrence of early life infections, maternal and paternal educational level, maternal and paternal smoking at home, the presence of pets at home, the region of residence (i.e., urban vs rural) and the country of infant's birth. Model 2 was further adjusted for the interaction between treatment arm and FHAD. The P-value derived from Poisson Generalized Estimating Equation (GEE) regression analysis and provides the treatment effect in the pHF compared to the SF group.

4. DISCUSSION

The present study indicates that infants with increased risk for CMPA (based on family history of allergy) may benefit, from an allergy point of view, from a combination of breastmilk and the studied infant formula with partially hydrolyzed whey protein. This combination resulted in a lower incidence of AD in a sub-population of infants with a positive family history for AD. Moreover, a trend was found towards a lower incidence of CMPA in the pHF group, as compared to EBF. The EBF group did not differ from SF in the incidences of AD and CMPA. In respect to growth, all groups showed small, negative Z-scores for body weight and BMI at baseline. Absolute growth improved during the study with the SF group showing the highest increase in body weight and BMI, while length improved the most in the pHF group. Z-scores for weight in the EBF and pHF groups were close together and developed in the same way during the study, with an initial decrease at the age of 4 months in EBF and improving thereafter.

The protective effect on certain allergy outcomes in the pHF group, was mainly observed in the mixed-fed infants, which represent the vast majority in that group in study's cohort. The number of exclusively formula fed infants was too small to draw any conclusion. In line with current study results, the GINI study also showed the beneficial effect of a hydrolyzed formula (61), while review studies even in the general population are indicative of the protective effect of pHF in not-fully breast-fed infants (Sauser, 2018). Studies on the role of early supplementation of intact CMP on later development of allergy-associated diseases are indicative of a protective effect, depending however on timing. Early CMP introduction, but not earlier than two weeks of life, has been associated with lower rates of IgE-mediated CMPA (48), while early as three days of life supplementation followed by complete CMP avoidance may result in opposite effects (70), (83). Even more, the timing for commencing pHF consumption is considered as important since the most beneficial effect in respect to allergy prevention is observed during the first 6 months of life (Vandenplas, 2019). It might be that EBF from birth onwards supplies too much allergenic B-lactoglobulin, whereas after an initial short period of 'no allergens' (extensively hydrolysed formula) a minimum number of allergens is necessary to build tolerance. Moreover, the minimum allergenicity albeit combined with residual antigenicity of proteins contained in the partially hydrolyzed formulas, might facilitate tolerance at least in a subpopulation of high risk for allergy infants (59).

The lower incidence of AD in the pHF group was noted in infants with a positive family history of AD. AD in the core family, has been long considered as a significant risk factor for development of any allergy-associated disease in the offspring, compared to any other allergic disease, as shown by epidemiological, intervention and genetic studies (61), (99). It is plausible, that the beneficial effect in the pHF compared to the SF group on AD and CMPA outcomes depend highly on the genetic background, which potentially modifies the preventive effect of a hydrolysate, as was previously suggested (GINI study).

In respect to growth outcomes, although increases in body weight and BMI Z-scores from baseline to 4 and 6 months were more pronounced for the SF group, respective length increases were significantly higher in the pHF group. It has been previously documented that the specific whey-based pHF formula used in our study, is non-inferior in respect to all infant growth outcomes compared to SF, although a margin of -3g/day was noted in a three-month intervention period for the pHF compared to the SF group (100), while review reports are confirmatory of the normal growth in infants consuming pHFs (101).

5. CONCLUSION

The data from the present study support that infants at high-risk for allergy who are not exclusively breastfed, may benefit from a nutritional intervention with a specific whey-based pHF complementary to breastfeeding compared to mixed-feeding with a standard formula. This combination reduced the development of AD during the first 6 months of life, in infants with a positive family history of AD. Supplementation of pHF formula to BF resulted in normal growth outcomes compared to EBF and SF fed infants.

6. STRENGTHS AND LIMITATIONS

The main strength of A.R.T. study is the double-blinded randomized controlled design. Moreover, only high-risk infants with a documented by a physician family history of allergy participated. Since subjects were recruited from public and private maternity hospitals/clinics in 3 different European countries providing a representative high-risk population sample, results could be generalizable. Infants had not consumed any formula with intact or partially hydrolyzed proteins prior to allocation and solid foods were allowed after the age of 4 months. All researchers were appropriately trained prior to initiation of the study, and centers were compared during training, to get the most accurate and comparable results. Furthermore, statistical analysis was performed by an independent third party.

Importantly, CMPA was objectively confirmed by open oral food challenge after an elimination diet in accordance with current CMPA guidelines. Although this could be considered as a strength of the study, one could see it as a limitation since the gold standard for food allergy diagnosis (double-blinded placebo-controlled food challenge) was not performed. It is generally accepted that the clinical diagnosis of AD in early life is challenging as the proposed criteria are difficult to apply given that they have been created for the assessment of older children. However, the AD outcome in ART was defined as a clinical diagnosis of AD by experienced clinicians supported by the objective SCORAD tool and the awareness CoMiSS tool.

Another limitation is the sample size and power estimation of the study that was based on limited data from older studies. The assumed incidence of CMPA was 20% (30) in the SF group, whereas in the actual study we found only 8%, which might suggest why the study did not detect full effectiveness of the pHF.

For the mixed feeding groups, breastmilk intake is estimated using an equation, in which the expected breastmilk intake per kg BW is calculated based on the age of the infant. From this amount the measured formula intake per kg BW is deducted. However, this method provides

sometimes negative values that were corrected and set to zero, which was in particular the case in the SF infants. In other words, this method only is a crude estimation of breast milk intake, subjected to bias.

Finally, true blinding for pHF is difficult due to its specific taste and smell as compared to SF (7, 12). Also working with FCA and FCB codes at the bottom of the tins was not ideal as it may lead to bias. However, products were prepared at home and FCA and FCB were nowhere explained during the study. Only a product developer at FrieslandCampina, not involved in the study, had access to decoding. Code-break or de-blinding did not take place until statistical analyses were completed.

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