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Breast milk poly unsaturated fatty acids: associations with adolescent allergic disease and lung function

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14 Short title- Breast milk PUFA and allergic disease

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1 **Abstract**

2 **Background**

3 It has been hypothesised that n-3 PUFA in breast-milk may assist immune and lung
4 development. There are very limited data on possible long-term effects on allergic disease
5 and lung function. The aim was to investigate associations of n-3 and n-6 PUFA levels in
6 colostrum and breast milk with allergic disease and lung function at ages 12 and 18 years.

7 **Method**

8 PUFAs were measured in 194 colostrum samples and in 118 three month expressed breast
9 milk samples from mothers of children enrolled in the Melbourne Atopy Cohort (MACS)
10 Study, a high risk birth cohort study. Associations with allergic diseases, skin prick tests and
11 lung function assessed at 12 and 18 years were estimated using multivariable regression.

12 **Results**

13 Higher levels of n-3 but not n-6 PUFAs in colostrum were associated with a trend towards
14 increased odds of allergic diseases, with strong associations observed for allergic rhinitis at
15 12 (OR=5.69[95%CI: 1.83,17.60] per weight%) and 18 years (4.43[1.46,13.39]) and eczema
16 at 18 years (9.89[1.44, 68.49]). Higher levels of colostrum n-3 PUFAs were associated with
17 reduced sensitisation (3.37[1.18, 9.6]), mean FEV₁ (-166ml [-332, -1]) and FEV₁/FVC ratio
18 (-4.6%, [-8.1,-1.1]) at 12 years.

19 **Conclusion**

20 Higher levels of colostrum n-3 PUFAs were associated with increased risks of allergic
21 rhinitis and eczema up to 18 years, and sensitisation and reduced lung function at 12 years.
22 As residual confounding may have caused these associations, they should be replicated, but
23 these results could indicate that strategies that increase maternal n-3 PUFA intake may not
24 aid in allergic disease prevention.

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27 Key words- allergic diseases, breast milk, colostrum, lung function, PUFA

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1 Key message- Introducing PUFA as a prevention strategy for allergic diseases should be
2 performed cautiously

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List of Abbreviations

MACS	- Melbourne Atopy Cohort Study
PUFA	- Polyunsaturated fatty acids
FVC	- Forced Vital Capacity
FEV ₁	- Forced Expiratory Volume in 1 second
MEF	- Mid Expiratory Flow rate
FEV ₁ /FVC Ratio	- The Ratio of FEV ₁ /FVC measurements
IQR	- Inter-quartile range
SD	- Standard Deviation
DHA	- Docosahexaenoic acid
EPA	-Eicosapentaenoic acid
DAG	-Direct Acyclic graph
SPT	-Skin Prick Test
RCT	-Randomised controlled trial

1 Introduction

2

3 The antenatal and neonatal periods are key times for immune system development (1).
4 Breast milk is the first food for most newborns. Along with many other nutritional and
5 bioactive factors, breast milk also contains poly-unsaturated fatty acids (PUFAs), which have
6 the potential to modulate the immune system (2). The n-3 and n-6 classes of PUFAs have
7 been identified as the most important for directing non-allergic and allergic responses via T-
8 helper cells, which may influence development of allergic phenotypes (3). PUFAs have a
9 variable length carbon chain and are classified according to the location of the first double
10 bond. In the n-3 class, the first double bond is between c-3 and c-4 and in the n-6 class
11 between c-6 and c-7 (from the methyl end). It is postulated that n-3 PUFAs are associated
12 with reduced inflammation by stabilizing the T cell membrane and production of less potent
13 inflammatory mediators compared with n-6 PUFAs (4).

14 A small number of randomised controlled trials (RCTs) have been undertaken examining the
15 effect of PUFA supplementation during early life on allergic disease outcomes. The effects
16 observed have lacked consistency, with some showing reduced risk with n-3 PUFA
17 intervention (5, 6) while others failing to show any effect (7). It appears that the outcomes
18 may vary depending on the timing of interventions, the PUFA dose, and the age of outcome
19 measurement. For example, one of the reasons underlying this inconsistency may be related
20 to differing exposure age (*in utero* versus post natal), with some trials supplementing
21 pregnant mothers (5, 8, 9) while in others the supplement was given post-natally to the
22 children (10-12). Furthermore, none of these studies have followed the participants into
23 adolescence. Hence, current evidence is limited and inconclusive.

24 Examining the associations between natural variation in breast-milk PUFA and allergic
25 disease outcomes in the child may help indicate the optimum level of PUFA for preventing
26 disease in early life. We have previously found that n-3 PUFAs in colostrum were associated
27 with increased risk of allergic sensitisation at 6 and 24 months of age in the Melbourne Atopy
28 Cohort Study (MACS) (13). Further, high levels of breast-milk n-3 PUFAs were associated
29 with an increased risk of non-atopic eczema, while higher levels of n-6 PUFAs in colostrum
30 were associated with an increased risk of childhood rhinitis (14) up to age 7 years. However,
31 early life allergic disease symptoms may not reflect the long term effects, including for

1 allergic rhinitis which is often not expressed until later teenage years. Using further data
2 from the Melbourne Atopy Cohort Study (MACS) we aimed to examine the associations
3 between colostrum or breast milk fatty acids and allergic diseases or lung function outcomes
4 to adolescence, to explore the long term associations with allergic disease and lung function.

1 **Materials and Methods**

2 **Study design and population**

3 Details of the MACS study design and sample have been described elsewhere (14). Briefly,
4 (n=620) pregnant mothers, attending Mercy Maternity Hospital antenatal clinics between
5 1990 and 1994 were recruited. Eligible children had at least one first degree relative with a
6 history of allergic disease (self-reported asthma, eczema, hay fever or severe food allergy).
7 MACS was initially a RCT to investigate three types of formula (cows' milk, soy or partially
8 hydrolysed whey formula) (15). The MACS mothers were un-blinded to the study formula
9 after the second birthday of their child. Similar to other studies of this kind, MACS has been
10 analysed as an observational birth cohort study for non-randomised exposures. The birth
11 cohort has subsequently been followed with assessments at ages 12 and 18 years.

12 The Mercy Maternity Hospital Ethics Committee provided initial approval and the Royal
13 Children's Hospital Ethics Committee approved the 18 year follow-up. Written informed
14 consent was obtained from mothers at recruitment and participants also provided individual
15 consent at the 18 years follow up.

16 **Data collection**

17 Baseline demographic details were obtained during the antenatal period. A research nurse
18 trained in the field of allergy conducted the survey by telephone every 4 weeks from birth to
19 64 weeks (including the details of the breastfeeding behaviour), at 78 weeks and at two years.
20 Annual follow-ups were conducted up to age 7 years, then at 12 and 18 years. The
21 International Study of Asthma and Allergies in Childhood (ISAAC questionnaire (16) was
22 administered at 12 and 18 years.

23 The colostrum samples were hand expressed and collected as close to delivery as possible
24 and breast milk samples were collected before the first morning feed and expressed using a
25 breast pump at approximately three months after delivery. When the quantity was inadequate,
26 additional volumes were collected at a subsequent feed. Initially the samples were frozen at -
27 20°C and thereafter at -80°C. Gas chromatography was used to analyse the fatty acid profile
28 according to the method of Bligh and Dyer (17). Further details of the fatty acid analysis
29 used for these samples have been described previously (13). Results are expressed as weight
30 percentage (wt%) for total n-3 and n-6 fatty acids.

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2 **Outcome definitions**

3 The primary outcomes were ISAAC definitions of current wheeze, allergic rhinitis and
4 eczema, measured at both 12 and 18 years (16).

5 **Current wheeze:** a response of “yes” to both “Have you ever had wheezing or whistling in
6 the chest?” and “Have you ever had wheezing or whistling in the chest in the past 12
7 months?” (16).

8 **Current allergic rhinitis:** a response of “yes” to both “Have you ever had a problem with
9 sneezing, or a runny, or a blocked nose when you did not have a cold or the flu?” and “Have
10 you ever had a problem with sneezing or a runny nose, or a blocked nose when you did not
11 have a cold or flu during the last 12 months?” (16).

12 **Current eczema:** a response of “yes” to both, “Have you ever had a problem with itchy
13 rash which was coming and going at least for a period of 6 months?” and “Have you ever had
14 a problem with itchy rash during the last 12 months?” and also the rash affecting at least one
15 of the following places – the folds of the elbow and/or behind the knees and/or in front of the
16 ankles and/or under the buttocks and/or around the neck ears or eyes (16).

17

18 **Skin prick testing**

19 Skin prick testing was performed at the 12 and 18 year follow-up visits. At 12 and 18 years
20 the allergens tested were cow’s milk, egg white, peanut, house dust mite (*Dermatophagoides*
21 *pteronysinus*), rye grass (*Lolium perenne*) and cat dander. At 18 years, additional allergens
22 were: *Alternaria tenuis*, *Penicillium notatum*, *Homodendrum cladosporides*, mixed grass
23 pollen, cashew and shrimp (ALK-Abello Horsholm, Denmark and Hollister-Stier, Spokane
24 WA, USA). A positive skin prick test was defined as a wheal size ≥ 3 mm and histamine
25 1mg/ml was used as the positive control. Details of the procedures are described elsewhere.

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3 **Lung function outcomes**

4 Pre bronchodilator spirometry was measured at both 12 and 18 years. Post bronchodilator
5 spirometry was measured only at 18 years. American Thoracic Society (1994) and American
6 Thoracic Society/European Respiratory Society guidelines (2005)(18, 19) were followed.
7 Participants were advised to abstain from short acting bronchodilators for four hours and long
8 acting bronchodilators for 12 hours before the procedure. At 12 years a Spirocard spirometer
9 was used (SpiroCard™ PC spirometer, QRS Diagnostic, Plymouth, MN, USA) and at
10 eighteen years an EasyOne™ (ndd Medical technologies Inc, Andover MA, USA) was used.
11 Anthropometric measurements were obtained at the time of spirometry (height to nearest 0.1
12 cm and weight to the nearest 0.1 kg).

13

14 **Statistical analysis**

15 Multivariable logistic regression was performed to investigate associations between the
16 different PUFA class levels (total n-3, n-6 and the n-3/n-6 ratio in both colostrum or breast
17 milk) and allergic or respiratory outcomes. Multivariable linear regression models were used
18 to investigate associations with lung function outcomes (FVC (ml), FEV₁(ml), MEF(ml/s)
19 and FEV₁/FVC(%)). A directed acyclic graph (DAG) was developed to identify potential
20 confounders for these associations (online repository figure 1). All analyses were adjusted
21 for maternal education (completed tertiary education), socioeconomic status (ANU3 scale
22 according to the father's occupation at baseline) (20), maternal history of smoking, maternal
23 history of allergic disease and the presence of older siblings as a priori potential confounders,
24 as was gender for its known association with the incidence of allergic disease. In addition, all
25 lung function models were further adjusted for gender, age and height at the time of
26 spirometry. Other potential confounders such as pets at home at the time of birth were
27 investigated. Only the variables that changed the effect estimates more than 10% were
28 retained in the final model. Potential non-linear associations were investigated using
29 fractional polynomials. Interaction models were fitted to check for effect modification by
30 maternal asthma, maternal atopy (≥ 3 mm on skin prick test), duration of breastfeeding and the
31 allocated formula group. For interaction models with breastfeeding, duration of breastfeeding

1 was classified as less or more than the median duration for exclusive breastfeeding (median 4
2 and 4.5 months for the mothers who provided colostrum and breast milk respectively), and
3 total duration of breastfeeding (median of 12 and 14 months for the mothers who provided
4 colostrum and breast milk respectively), among the mothers who provided colostrum or
5 breast milk samples.

6
7 In this cohort higher levels of n-3 PUFAs (Poly unsaturated fatty acids) were associated with
8 increased risk of early life sensitisation (13). We therefore, tested if any association between
9 n-3 PUFAs and later outcomes might be due to this effect on early life sensitisation. To do
10 this, a mediation analysis was performed, using the “medeff” module in Stata, to test if any
11 associations between breast milk PUFA and the outcomes may be due to indirect effects
12 through early life sensitisation (at 6 months or 24 months a positive SPT for any of the tested
13 allergens) (21).

14 Estimates are presented as odds ratios (OR) and 95% confidence intervals (CI) for allergic
15 disease or sensitization outcomes and beta coefficients for the lung function outcomes,
16 expressed per 1% increase in weight of PUFA. All statistical analyses were performed using
17 STATA software (Version 13, Stat Corp, College Station, TX, USA).

1 Results

2 A total of 224 women provided either colostrum within the first 3 days postpartum (n=194)
3 and/or breast milk (n=118) at approximately three months postpartum, with 88 providing
4 both samples. The mean duration of exclusive and any breastfeeding was 14.3 weeks
5 (SD=8.8) and 48.6 weeks (SD=26.9) respectively for participants providing colostrum and
6 15.5(8.6) and 55.1(23.6) for participants provided a sample of breast milk.

7 While the demographics of mothers who provided breast milk and those who did not were
8 similar on a range of factors (table1), mothers who donated a colostrum sample were less
9 likely to be atopic and more likely to have an older child. Mothers who donated a 3-month
10 breast-milk sample had higher socioeconomic status (table 1) (14). Similar to other published
11 data, the level of n-3 PUFAs observed was much lower than that of n-6 PUFAs in both
12 colostrum and breast-milk (table 2) (22). In those mothers who provided both a colostrum
13 and breast-milk sample, there was a moderate correlation between n-3 PUFA ($r=0.51(95\%CI:$
14 $0.333, 0.648)$ $p<0.001$), between these two times. A similar correlation was observed for n-6
15 PUFA ($0.58(95\%CI: 0.422, 0.704)$ $p<0.001$). Details of individual fatty-acid levels have been
16 previously published (13, 14).

Table 1- Distribution of demographic factors among the mothers who did and did not provide milk samples

Demographic factors	Colostrum			Breast milk		
	Yes n=194	No n=426	P value	Yes n=118	No n=502	P value
Mean Duration of Exclusive breastfeeding, weeks (SD)	14.3(8.8)	11.5(9.1)	0.58	15.5(8.6)	11.6(9.1)	0.45
Mean Duration of Total breastfeeding, weeks (SD)	48.6(26.9)	38.6(28.4)	0.28	55.1(23.6)	38.7(28.4)	0.09
Maternal atopy (83% (514/620))	77.3(150/194)	85.4(364/426)	0.01	78.8(93/118)	83.8(421/502)	0.19
Maternal asthma (43%(267/620))	40.9(79/193)	44.3(188/424)	0.48	39.8(47/118)	44.1(220/499)	0.43
Older sibling (60%(369/618))	63.4(123/194)	58.0(246/424)	0.02	59.3(70/118)	59.8(299/500)	0.84
Male child (51%(317/620))	52.1(101/194)	50.7(216/426)	0.82	50.8(60/118)	51.9(257/502)	0.84
Mean of Socioeconomic status (SD)	47.4(19.23)	45.3(21.1)	0.64	50.1(18.5)	44.9(20.9)	0.01
Maternal education (41%(256/620))	42.7(83/194)	40.6(172/424)	0.64	37.3(44/118)	42.2(211/500)	0.32
Maternal smoking (25.6%(159/620))	9.58(38/194)	62.4(121/426)	0.07	20.33(24/118)	26.9(135/502)	0.67
Pets at home (97.3%(603/620))	51.6(98/190)	53.9(223/413)	0.61	45.8(54/118)	55.1(267/485)	0.63
Allocation cow' milk group	34.5(67/194)	32.6(139/426)	0.77	31.3(37/118)	33.6(169/502)	0.81
Allocation soy group	40.2(78/194)	30.5(130/426)	0.24	39.8(47/118)	32.0(161/502)	0.37
Allocation pHWF group-	25.2(49/194)	36.8(157/426)	0.15	28.8(34/118)	34.2(172/502)	0.49

*Duration of breastfeeding measured in weeks *Socioeconomic status measured according to the ANU3 scale (scaled 0-100 according to father's occupation) *

Maternal education as completion of tertiary education *Maternal smoking as ever smoking

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Table 2- Distribution of **total fatty acid** concentrations in **colostrum and breast milk**

Colostrum (n=194)		
PUFAs	Median (Inter-quartile range)	Range
n-6 wt%	14.0 (11.9,16.6)	7.96-32.72
n-3 wt%	1.8 (1.6,2.1)	1.12-2.82
n-6/n-3 ratio	7.6 (6.3,9.1)	4.35-27.07
Breast milk (n= 118)		
PUFAs	Median (Inter-quartile range)	Range
n-6 wt%	15.9 (12.8,20.2)	7.05-30.89
n-3 wt%	1.6 (1.4,1.8)	0.82-2.99
n-6/n-3 ratio	9.5 (7.9,12.6)	4.72-26.50

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3 **Association between colostrum or breast milk fatty acids and wheeze, eczema and**
 4 **allergic rhinitis at 12 and 18 years**

5 There was a general trend towards increased odds of allergic diseases with increased
 6 colostrum n-3 levels (table 3). These increased odds were strongest for the associations with
 7 allergic rhinitis at 12 years (OR=5.69, 95% CI= 1.83,17.62) and at 18 years for eczema
 8 (OR=9.89,95%CI=1.43,68.49) and allergic rhinitis (OR=4.43, 95%CI=1.46,13.39).

9 Furthermore, increasing n-6 PUFA in colostrum was associated with increased odds of
 10 eczema (OR=1.22, 95%CI:1.04,1.44) at 18 years of age. We did not observe any
 11 associations with n-3/n-6 ratio and with breast milk fatty acids. There was no evidence of
 12 non-linearity for these associations, or for any of the other outcomes, and nor was there
 13 substantial differences with the unadjusted associations (online repository table 2)

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Table 3- Adjusted* associations between n-3 & n-6 PUFA concentrations in colostrum/breast milk and disease outcomes at 12 and 18 years using the ISAAC definitions of outcomes.

Associations with colostrum fatty acid levels

Disease outcomes at 12 years						
	Wheeze (n=33/131)		Eczema (n=14/115)		Allergic rhinitis (n=58/132)	
Fatty acid wt%	OR	95%CI	OR	95%CI	OR	95%CI
Total n-6	0.99	0.89,1.11	0.95	0.78,1.16	0.97	0.88,1.07
Total n-3	1.70	0.54,5.29	2.90	0.52,16.08	5.69	1.83,17.6
n-6/n-3	0.95	0.86,1.12	0.77	0.54,1.10	0.86	0.74,1.00
Disease outcomes at 18 years						
	Wheeze (n=31/140)		Eczema (n=16/140)		Allergic rhinitis (n=67/139)	
Fatty acid wt%	OR	95%CI	OR	95%CI	OR	95%CI
Total n-6	0.96	0.85,1.08	1.22	1.04,1.44	1.06	0.96,1.18
Total n-3	3.44	0.97,12.13	9.89	1.43,68.49	4.43	1.46,13.39
n-6/n-3	0.88	0.74,1.06	1.11	0.94,1.32	0.99	0.87,1.12
Associations with breast milk fatty acid levels						
Disease outcomes at 12 years						
	Wheeze (n=21/76)		Eczema (n=5/71)		Allergic rhinitis (n=29/76)	
Fatty acid wt%	OR	95%CI	OR	95%CI	OR	95%CI
Total n-6	0.96	0.86,1.07	0.97	0.74,1.26	0.98	0.89,1.09
Total n-3	0.76	0.18,3.17	0.68	0.04,13.02	1.31	0.39,1.39
n-6/n-3	0.99	0.87,1.12	0.92	0.65,1.29	0.99	0.88,1.11
Disease outcomes at 18 years						
	Wheeze (n=17/88)		Eczema (n=14/88)		Allergic rhinitis (n=40/88)	
Fatty acid wt%	OR	95%CI	OR	95%CI	OR	95%CI

Total n-6	0.96	0.85,1.09	1.01	0.88,1.15	1.03	0.94,1.14
Total n-3	0.45	0.09,2.18	3.33	0.54,20.27	0.76	0.24,2.44
n-6/n-3	1.02	0.89,1.16	0.90	0.75,1.09	1.05	0.94,1.16

*Adjusted for gender, maternal smoking status, maternal education, maternal socioeconomic status, presence of any siblings and the maternal history of the disease outcome concerned in the proband

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1 **Association between colostrum or breast milk fatty acids and allergic sensitisation at 12**
2 **and 18 years**

3 Increasing n-3 PUFA in colostrum was associated with increased sensitisation at 12 years
4 (OR=3.37, 95%CI=1.18, 9.60) with a similar but statistically non-significant association at 18
5 years (OR=2.77, 95%CI=0.81,9.41, online repository table 1). Breast milk PUFA n-3 and n-6
6 were not associated with sensitisation outcomes (online repository table 1).

7

8 **Association between colostrum or breast milk fatty acids and lung function outcomes at**
9 **12 and 18 years**

10 At 12 years, 60% of participants underwent lung function assessment (median age =11.5
11 years, IQR=10.0-12.9) and 66% were tested at 18 years. There was some evidence that
12 increasing levels of n-3 PUFAs in colostrum were associated with reduced pre
13 bronchodilator FEV₁ (estimated mean difference of -166, 95%CI:-332,-1 ml per wt%
14 increase in n-3 PUFA) and FEV₁/FVC ratio (-4.6%, 95%CI: -8.1,-1.1) at age 12. Non-
15 significant reductions in the same spirometric parameters were also observed at 18 years for
16 n-3 PUFA in colostrum (table 4).

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Table 4 – The adjusted* associations between n-3 & n-6 PUFA concentrations in **colostrum** and **spirometry outcomes at 12 and 18 years**

Associations with colostrum fatty acid levels						
12 years pre bronchodilator spirometry (n=132)						
wt%	FVC (ml)		FEV₁ (ml)		FEV₁/FVC ratio (%)	
	β	95%CI	β	95%CI	β	95%CI
Total n-6	5	-16,26	6	-11,23	0.1	-0.2,0.4
Total n-3	-59	-268,150	-166	-332,-1	-4.6	-8.1,-1.1
n-6/n-3	11	-14,35	16	-34,36	0.3	-0.2,0.7
18 years pre bronchodilator parameters (n=136)						
wt%	FVC (ml)		FEV₁ (ml)		FEV₁/FVC ratio (%)	
	β	95%CI	β	95%CI	β	95%CI
Total n-6	-4	-29,22	-3	-25,19	0	-0.3,0.4
Total n-3	-36	-307,235	-197	-421,40	-3.1	-6.8,0.7
n-6/n-3	-5	-35,25	6	-20,32	0.2	-0.2,0.6
18 years post bronchodilator parameters (n=131)						
wt%	FVC (ml)		FEV₁ (ml)		FEV₁/FVC ratio (%)	
	β	95%CI	β	95%CI	β	95%CI
Total n-6	-3	-28,23	-4	-28,20	0	-0.3,0.3
Total n-3	-60	-336,216	-167.9	-418,82	-2.2	-5.6,1.2
n-6/n-3	-3	-33,28	2.48	-25,30	0.1	-0.2,0.5

1 * Adjusted for gender, age and height at the time of spirometry, mothers education status,
 2 socioeconomic status of the family, maternal history of smoking, maternal asthma and any
 3 elder siblings

4

5

6 The only association found between breast milk fatty acids and lung function outcomes at 18
 7 years was the association between total n-3 PUFA levels and reduced mean post
 8 bronchodilator FVC (-386ml, 95%CI:-725,-47) (table 5).

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Table 5 – The adjusted* associations between n-3 & n-6 PUFA concentrations in **breast milk** and **spirometry outcomes at 12 and 18 years**

Associations with breast milk fatty acid levels						
12 years pre bronchodilator parameters (n=77)						
wt%	FVC (ml)		FEV1 (ml)		FEV1/FVC ratio (%)	
	β	95%CI	β	95%CI	β	95%CI
Total n-6	2	-17,20	8	-7,24	0.2	-0.1,0.5
Total n-3	-5	-278,189	-92	-288,104	-1.5	-4.9,1.9
n-6/n-3	8	-14,31	14	-5,33	0.2	-0.2,0.5
18 years pre bronchodilator parameters (n=84)						
wt%	FVC (ml)		FEV1 (ml)		FEV1/FVC ratio (%)	
	β	95%CI	β	95%CI	β	95%CI
Total n-6	-4	-29,22	4	-16,23	0.2	-0.2,0.5
Total n-3	-256	-582,71	-63	-317,192	3.5	-7.6,7.8
n-6/n-3	4	-26,33	1	-21,24	-0.1	-0.5,0.3
18 years post bronchodilator parameters (n=81)						
wt%	FVC (ml)		FEV1 (ml)		FEV1/FVC ratio (%)	
	β	95%CI	β	95%CI	β	95%CI
Total n-6	-6	-33,20	1	-22,24	0.2	-0.1,0.5
Total n-3	-386	-725,-47	-248	-544,48	2.1	-1.8,6.1
n-6/n-3	10	-21,41	11	-15,38	0.1	-0.3,0.4

1 * Adjusted for gender, age and height at the time of spirometry, mothers education status,
 2 socioeconomic status of the family, maternal history of smoking, maternal asthma and any
 3 elder siblings

4

5 There was no evidence of important effect modification by maternal asthma, maternal atopy
 6 or the allocated formula group (results not shown). The mediation analysis revealed that
 7 none of the observed associations were mediated via early life sensitization (at either six or
 8 24 months results not shown).

1 There was limited evidence that the associations between breast milk PUFA and allergic
2 disease outcomes were modified by duration of exclusive or total breastfeeding. While we
3 observed moderate statistical evidence for some interactions (online repository tables 4, 5,6
4 and 7), these were inconsistent and need to be interpreted cautiously due to the multiple
5 comparisons we have performed.

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1 Discussion

2 In this high risk cohort followed up to 18 years of age, we found that increased n-3 PUFA in
3 colostrum was associated with: i) increased odds of allergic rhinitis, eczema, and
4 sensitisation; and ii) reductions in FEV₁ and FEV₁/FVC ratio only at 12 years with similar
5 but non-significant reductions in spirometric parameters at 18 years. While these results are
6 consistent with previous findings from this cohort, in which we reported that n-3 PUFAs in
7 colostrum were associated with an increased risk of a positive SPT at 6 and 24 months of
8 age(13), they are not consistent with the hypothesis that early life exposure to n-3 PUFA may
9 reduce risk of allergic diseases. We did not observe any significant associations with breast
10 milk PUFAs (total n-3, n-6 or n-6/n-3). Moreover, none of the observed associations were
11 mediated via early life sensitization. Although we tested for interactions with the duration of
12 breastfeeding, we had limited statistical power to observe such effects, and there was no clear
13 pattern of associations. .

14 The antenatal and neonatal periods are believed to be important in the development of the
15 immune system (23). Immune programming in early life is critical in developing immune
16 competence and to avoid development of atopy and immune mediated disorders including
17 asthma (23). Here, PUFAs in colostrum were more strongly related to allergic outcomes
18 than PUFAs in three month breast milk, which may indicate a critical window in very early
19 life where there is potential immune modulation by exposure to n-3 PUFAs (24). In contrast
20 to our findings, some studies have observed that breast milk n-3 was associated with a
21 reduction of allergic disease outcomes (25, 26). It is also possible that colostrum fatty acids
22 are an indicator of maternal dietary intake during pregnancy, which has influenced the
23 infant's immune maturation in utero. At the time of recruitment of mothers to the MACS
24 project, fish and fish oil supplementation was not widely recognised as a potential mechanism
25 for prevention of allergic diseases, so the associations that we have observed are unlikely to
26 be confounded by degree/severity of family history of allergic disease. Furthermore, the
27 observed associations cannot be due to reverse causation, as the outcomes in the child were
28 not known at the time of the colostrum sample collection, so modification of the maternal diet
29 due to signs of illness in the child was not possible.

30 Several observational studies assessed associations between breast milk PUFA and allergic
31 diseases and the results have been mixed, possibly due to methodological issues. Among the
32 birth cohort studies which assessed eczema as an outcome (14, 27-31), two studies found that

1 n-3 PUFA was protective, while n-6 PUFA and the ratio were not associated with eczema.
2 The evidence is also mixed for sensitization with n-3 PUFA protective in some (27, 29) and
3 even n-6 PUFA protective among children of mothers without allergy (30). In contrast, n-3
4 PUFA was protective on wheeze/asthma in children of mothers with allergy (29) and n-6
5 PUFA had detrimental effects (22, 29). The age at which the outcomes were measured may
6 also be an important factor. Only one other study has assessed the outcomes in late childhood
7 and adolescence, once the immune system is fully mature (29). Finally, studies in this area
8 have generally had limited sample sizes (varying between 30–352 participants), which may
9 have resulted in a lack of statistical power to detect associations.

10 A key issue with the evidence is how potential confounding has been addressed. Among the
11 birth cohort studies, most (13, 14, 22, 29, 32) have adjusted for confounding factors but
12 others have not (26, 31, 33). Although studies with unadjusted estimates may be subject to
13 uncontrolled confounding, even those studies that have used statistical adjustments have not
14 demonstrated consistent results (22, 25). Over-adjustment may have also affected these results,
15 with some studies adjusting for factors, such as vaccination schedule (25) or smoking of the
16 child at 14 years of age (29), which cannot possibly be a confounding factor on these
17 associations. As with all observational studies, including our own, residual confounding by
18 unmeasured factors may have influenced the observed associations.

19 RCTs which have supplemented PUFAs during pregnancy and lactation, have also produced
20 mixed results (5-8, 34). Based on two studies (n=823) (35) that supplemented mothers with
21 high doses of n-3 PUFA (6) (36), a recently published Cochrane review identified that the
22 children of women supplemented with n-3 during pregnancy or lactation had reduced risk of
23 IgE mediated allergy, sensitization, and IgE mediated eczema, but there was no effect on
24 allergic rhinitis or asthma (35). The RCTs that have supplemented children (rather than
25 mothers) directly with n-3 PUFAs have produced heterogeneous results. One study observed
26 a lowered immune response and reduced allergic diseases, via suppressing Th2, when the
27 intervention was from birth to six months of age (37). In contrast, an Australian study that
28 supplemented infants from 6 months of age with 184 mg n-3 PUFA daily observed a
29 reduction in cough (38-40), but no effect on asthma/wheeze, eczema or sensitisation. This is
30 the only study to date to have reported associations with lung function outcomes (measured at
31 the age of five years), and again no effect of n-3 supplementation was observed (40).

1 It is not possible to directly compare the results from intervention trials and observational
2 studies of breast milk PUFA. Supplementation during the antenatal period will impact on the
3 colostrum levels of PUFA. If supplementation ceased at time of delivery, these levels are
4 likely to decline, and breast milk will reflect post-partum dietary intake. While
5 supplementation of diet with either oily fish or capsules has a similar effect on plasma levels
6 of n-3 (41), the dose of PUFA given in the intervention trials is much higher than could
7 typically be achieved by dietary intake.

8 It remains unclear why our observational study suggests that n-3 PUFA occurring naturally in
9 colostrum or breast milk is related to higher risk of eczema, allergic rhinitis and sensitisation,
10 and lower FEV₁, while supplementation trials suggest a reduced risk of IgE mediated disease,
11 at least in early life. It is possible, that differences in the bioavailability between breast milk
12 and supplemental PUFA could have contributed to such differences. Furthermore, animal
13 studies revealed that n-3 PUFA can increase the susceptibility of allergic diseases by
14 inhibiting production of Th1-type cytokines with little effect on Th2-type cytokines (42).
15 Other possible protective mechanisms include reduction of Th2 and IgE responses via PGE2
16 production (43), as well as the anti-inflammatory effects of resolving and protectins (44).

17 There are other determinants of maternal PUFA levels beyond diet, including genetically
18 determined variation in fatty acid metabolism (45). Plasma concentration of PUFA is affected
19 by fatty acid desaturase enzyme concentrations in the liver (46). Furthermore, colostrum and
20 breast milk PUFA levels may vary depending on the concentration of membrane bound fatty
21 acid metabolic enzymes in the mammary glands (46). Therefore, PUFA levels in breast milk
22 are not only dependent on maternal diet but also maternal fatty acid metabolism (47). The
23 genetic and metabolic variations that lead to variability in maternal fatty acid levels may also
24 influence the risk of allergic disease and lung function in the offspring.

25 **Strengths & Limitations**

26 Our study has a number of important strengths. MACS mothers were encouraged to
27 breastfeed and the allocated formula was utilised when the mother had decided to cease or
28 partially cease breastfeeding. High rates of breastfeeding were achieved, making this an ideal
29 cohort to study associations of early life breast milk PUFA exposure and allergic disease
30 outcomes in later life. We have reported outcomes up to 18 years, the longest follow-up to
31 date for published studies assessing these associations, and the associations between n-3
32 PUFA and increased risk of allergic sensitisation and disease are consistent across the various

1 age periods. Prospective collection of data from this cohort allowed us to investigate and
2 adjust for a number of potential confounding factors, including markers of socio-economic
3 status and maternal history of allergic disease. We also found no evidence that duration of
4 breastfeeding, maternal history of asthma, atopy or formula intervention group modified
5 these associations. It should be noted however, that we had limited statistical power to detect
6 such effects. Additionally we were able to investigate PUFAs at two different time points,
7 having both colostrum and three month breast milk samples.

8 A number of limitations need to be considered when interpreting these results. These results
9 may not be generalizable to the general population as MACS is a high risk cohort.
10 Furthermore, as only a limited number of women from the MACS provided breast milk
11 samples and there were further losses to follow-up at the 12 and 18 years visits, we had
12 limited statistical power to detect associations. Since many women only provided a breast
13 milk sample or a colostrum sample, it was not possible to directly compare associations with
14 the fatty acids at each time point. Also, some maternal factors and delivery details that may
15 be important in understanding these associations were not collected within this study.
16 Specifically, we were unable to assess the influence of maternal diet during pregnancy,
17 maternal BMI or mode of delivery, on these associations and also we do not have data on
18 number of breast milk collection times that was carried out to obtain the required volume.
19 Furthermore, we do not have food frequency questionnaires for MACS participants, therefore
20 we do not know whether these children have been supplemented or fed with more PUFA
21 compared to the general population as this group of children are at a high risk for allergic
22 diseases. It should be noted that the allocated formula, or any other commercially available
23 formula at the time of this study, was not fortified with n-3. Furthermore, mothers were
24 unaware of the allocated formula during breastfeeding, making it unlikely that there would
25 have been a systematic difference in the consumption of n-3 rich foods during lactation.
26 Breast milk PUFA was measured at two time points, which may not reflect the concentration
27 over the entire period of lactation. There were substantial, but not perfect, correlations
28 between the PUFA levels at these two time points. Finally, as this is an observational
29 analysis, there is the potential for residual confounding to have impacted on these findings as
30 it may not reflect the whole duration of breastfeeding.

31

32

1 **Conclusion**

2 In summary, high maternal n-3 PUFA levels in colostrum might be detrimental rather than
3 hoped for beneficial for children's lung function and increase the risk of allergic diseases.
4 While n-3 PUFA may be important for other biological functions (48), these results suggest
5 that simply adding n-3 PUFAs to the maternal and infant diet, especially in the context of a
6 family history of allergy, may not reduce the risk that the child will develop allergic
7 sensitisation and disease, or improve lung function. Larger studies are necessary to better
8 understand the relationship between early life dietary fatty acids modification on long term
9 allergic-respiratory health outcomes.

10

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21

22

23 **Competing interests**

24 None.

25

26

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5
6

7 **Author contribution**

8 Melbourne Atopic Cohort Study- S.C.D, A.J.L, K.J.A, M.J.A, C.S and C.J.L designed,
9 obtained funding and conducted the Melbourne Atopic Cohort Study. R.S and F.T obtained
10 funding to perform the breast milk fatty acid analysis.

11 N.T.W, A.J.L and J.A.S led the analysis of data.

12 All authors contributed to interpreting of the data, drafting the manuscript, to the intellectual
13 content and for the revising of the final draft of the manuscript. The final version of the
14 manuscript was approved by all the authors.

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