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EXPERIMENTAL ALLERGY AND IMMUNOLOGY

# Differences in exosome populations in human breast milk in relation to allergic sensitization and lifestyle

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## Keywords

allergic sensitization; anthroposophic lifestyle; breast milk; exosomes.

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## Abstract

**Background:** Breast-feeding has many beneficial effects on the developing immune system of the newborn. Breast milk contains immunoregulatory factors, such as nano-sized vesicles named exosomes. This study aimed at characterizing breast milk exosomes from human early milk and mature milk and to investigate whether allergic sensitization and an anthroposophic lifestyle could influence the exosome profile.

**Methods:** Breast milk was collected from 22 mothers at day 3–8 and from 61 mothers at 2 months postpartum, all part of the ALADDIN birth cohort. Isolated exosomes were captured on anti-MHC-class II- or anti-CD63 beads and analyzed by flow cytometry. Exosomal phenotype was related to lifestyle and allergic sensitization of the mothers, and sensitization of the child at 2 years of age.

**Results:** We found a higher content of exosomes in early milk compared with mature milk. Early milk exosomes were enriched in HLA-DR molecules and displayed significantly lower levels of HLA-ABC compared with those in mature milk. Phenotypically different subpopulations of exosomes were found in mature milk. Significantly lower levels of MUC1 were detected on CD63-enriched exosomes from sensitized mothers compared with nonsensitized. Furthermore, women with an anthroposophic lifestyle had significantly lower MUC1 expression on their HLA-DR-enriched milk exosomes and up-regulated levels of CD63 on CD63-enriched exosomes compared with nonanthroposophic mothers. Notably, mothers whose children developed sensitization had an increased amount of HLA-ABC on their milk exosomes enriched for CD63.

**Conclusions:** The phenotype of exosomes in breast milk varies with maternal sensitization and lifestyle, which might influence allergy development in the child.

Several studies have tried to elucidate whether breast milk can influence allergy development in the child (1). Conflicting results exist probably due to that different studies have been performed in different environments, with subjects of different genetic background and lifestyles. An anthroposophic lifestyle has been shown to be associated with a lower prevalence of allergic sensitization (2–4) and is characterized by

several features such as home deliveries (4), an organic diet rich in live lactobacilli, and a restricted use of antibiotics, antipyretics, and vaccines (3).

Breast milk contains many components that promote development of neonatal immune competence, such as antibodies, cytokines, and immune cells including antigen-presenting cells (APC) and T cells (5). Exosomes, 30–100 nm in size, originate from the endosomal compartment and are released from different cells (6), but their functions *in vivo* are far from being understood. Exosomes from tumor cells can inhibit the function of immune cells (7), while exosomes

## Abbreviations

APC, antigen-presenting cells; MUC1, mucin-1; NTA, nanoparticle tracking analysis; PBMC, peripheral blood mononuclear cells.

from APC can stimulate antitumor immune responses (8). We have earlier proposed that exosomes might contribute to the allergic reaction, for example, by APC-derived exosomes activating Th2 cells (9, 10), and we have shown the presence of proinflammatory exosomes in bronchoalveolar lavage fluid of asthmatic patients (11). Previously, we revealed the ability of human breast milk exosomes to induce Foxp3 expression in autologous and allogeneic peripheral blood mononuclear cells (PBMC) *in vitro* (12), suggesting that exosomes in breast milk have the potential to influence the immune system of the infant. Since then, others have confirmed the presence of milk exosomes in other species (13, 14), and we and others have shown that human milk exosomes also contain RNA (15, 16). Although maternal allergies (17, 18) as well as environmental factors (19) seem to influence the immunological composition in milk, it is still debated whether these differences affect the allergic outcome in the child.

As an anthroposophic lifestyle is associated with a reduced risk of allergic disease in children (2–4), we here sought to assess whether this lifestyle as well as maternal sensitization influences the composition of breast milk exosomes and whether these differences associate with allergic sensitization of the child at 2 years of age.

## Material and methods

### Subject characterization

This study is based on the prospective birth cohort Assessment of Lifestyle and Allergic Disease During Infancy (ALADDIN), which focus on elucidating the impact of lifestyle and environmental factors during pregnancy and childhood on the development of allergic disease (4). Mothers included in this paper were a subgroup of nonsmokers, who did not give formula to their child during the first days of life and where sufficient quantities of breast milk could be obtained. Families were classified into different lifestyle groups based on answer to three questionnaire questions: (i) 'What kind of preschool/school will your newborn child probably go to?' (ii) 'Has any of the parents, no matter which type of school you have planned for your child, an anthroposophic view of life?', and (iii) 'Is the family's everyday life influenced by an anthroposophic view of life?'. Families were defined as anthroposophic if they answered 'anthroposophic' to question 1 and 'yes' to questions 2 and 3. Families answering 'nonanthroposophic' to question 1 and 'no' to questions 2 and 3 were classified as nonanthroposophic. The Research Ethical Committee at Huddinge University Hospital, Stockholm, Sweden, approved the study, and written informed consent was obtained from all families.

### Definition of sensitization

Parental IgE sensitization was defined by ImmunoCAP Phadiatop® (Phadia AB, Uppsala, Sweden) containing a mixture of 11 inhalant allergens. Blood from the children at 2 years of age was analyzed by ImmunoCAP® (Phadia AB) for seven common allergens (hen's egg, cow's milk, peanut,

cat, dog, birch, and timothy). Parents were considered sensitized if IgE levels were  $\geq 0.35$  kU<sub>A</sub>/l in Phadiatop® and for the children for at least one of the seven allergens.

### Collection of breast milk

For paired milk sample analysis, early milk from 22 mothers ( $30.7 \pm 4$  years, mean  $\pm$  SD) was collected at day 3–8 ( $5 \pm 1.3$  days) and at 2 months (mature milk) postpartum using a manual pump. To address differences in mature milk with regard to maternal sensitization or lifestyle of the mother, we used mature milk from 47 mothers (Table 1), including mature milk from 8 of the mothers used for the paired milk sample analysis who fulfilled all the criteria of either anthroposophic ( $n = 3$ ) or nonanthroposophic ( $n = 5$ ). In total, milk from 61 mothers was analyzed in any of the figures. Early milk ( $n = 22$ ) and mature milk ( $n = 61$ ) were kept at  $+4^{\circ}\text{C}$  until frozen within 24 h and stored at  $-80^{\circ}\text{C}$  for a maximum of 4 years.

### Purification of breast milk exosomes

Exosomes from breast milk were purified by a series of centrifugations, filtrations, and ultracentrifugations as previously described (12). The 100 000 g pellet was diluted in a small volume of PBS. Exosomal protein concentration was measured by a DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). For sucrose gradient purification, exosomes were layered on top of a sucrose density gradient (0.25–2 mM sucrose and 20 mM HEPES, Sigma-Aldrich, St Louis, MO, USA) and ultracentrifuged at 79 000 g at  $4^{\circ}\text{C}$ .

**Table 1** Demographic data on the study population of parents

| Characteristics                         | Anthroposophic<br><i>N</i> = 23 | Nonanthroposophic<br><i>N</i> = 24 | <i>P</i> value |
|-----------------------------------------|---------------------------------|------------------------------------|----------------|
| Parents                                 |                                 |                                    |                |
| Mothers sensitized                      | 11/23 (47.8)                    | 10/24 (41.7)                       | 0.772          |
| Fathers sensitized                      | 7/23 (30.4)                     | 4/24 (16.7)                        | 0.318          |
| Age of mother at birth of child (years) | $32.2 \pm 7.2$                  | $30.8 \pm 3.9$                     | 0.397          |
| Delivery                                |                                 |                                    |                |
| Home delivery                           | 9/23 (39.1)                     | 1/24 (4.2)                         | 0.004          |
| Acute cesarean section                  | 2/23 (8.7)                      | 1/23 (4.3)                         | 0.742          |
| Planned cesarean section                | 3/23 (13.0)                     | 2/23 (8.7)                         |                |

Continuous variables presented with mean and SD and tested using independent *t*-test.

Categorical variables presented with *n/N* and % and tested using Fisher's exact test.

for 20 h using a SW 28 rotor (Beckman Coulter, Brea, CA, USA).

### Nanoparticle tracking analysis (NTA)

Size distribution and particle concentration in breast milk-exosome preparations were analyzed by measuring the rate of Brownian motion using a NanoSight LM10 system equipped with a fast video capture and particle-tracking software NTA 2.2 (NanoSight, Amesbury, UK). Exosome preparations were diluted in PBS (1 : 3000–1 : 5000), and particle concentration was normalized to milk volume.

### Flow cytometry analysis of exosomes

Exosomes were adsorbed onto 4.5  $\mu\text{m}$   $\varnothing$  dynabeads precoated with anti-MHC-class II MoAbs clone HKB1 (Invitrogen Ltd, Paisley, UK) or 4  $\mu\text{m}$   $\varnothing$  latexbeads precoated with anti-CD63 MoAbs clone H5C6 (BD Biosciences, Erembodegem, Belgium) and incubated overnight as previously described (12). Exosomes corresponding to 1.5 ml of original milk volume were coated on 1  $\mu\text{l}$  beads ( $\sim 4 \times 10^5$  dynabeads,  $\sim 1.2 \times 10^6$  latexbeads) and stained with FITC-conjugated anti-HLA-DR, anti-HLA-ABC, anti-MUC1 (BD Biosciences), anti-CD36 (Beckman Coulter, Bromma, Sweden) or PE-conjugated anti-CD63 or anti-CD81 (BD Biosciences) and compared with isotype controls. Single beads were gated,  $5 \times 10^3$  events were acquired per sample in a FACS Calibur (BD Biosciences), and data were analyzed using CellQuest Pro software (BD Bioscience).

### Statistical analysis

The early milk *vs* mature milk samples and differences in subpopulations of exosomes were analyzed using dependent t-test. Continuous outcomes (milk) were analyzed with ANOVA with sensitized mother and lifestyle as main effects in the model, and logistic regression was used for the dichotomous outcomes (sensitization of the child). All continuous variables were log-transformed ( $\log_{10}$ ) prior to analysis to achieve normality. The assumptions for the models with continuous outcomes, normality and homoscedasticity, were visually inspected and tested with the Shapiro–Wilk and the Breusch–Pagan test, respectively, and the assumptions met (after log-transformation). Data were adjusted for lifestyle when comparing the allergic sensitization of the mother or adjusted for sensitization when comparing lifestyle. Logistic regression was performed and corrected for allergic sensitization and lifestyle of the mother when looking at the sensitization of the child as outcome. Due to our small sample size, additional variables were not adjusted for in the analyses. In the logistic regressions, linearity was investigated by modeling the respective milk variables with restricted cubic splines and by plotting the functional form. The relationship between the milk variables and allergic sensitization at 2 years of age was considered linear. All analyses were performed in R v 2.15.3 (R Foundation for Statistical Computing, Vienna, Austria). Data presented in the tables were analyzed using Fisher's

exact test. The level of significance was set to 5%, two-sided, for all tests, and no adjustment for multiplicity was performed.

## Results

### Differences between milk exosomes from early milk and mature milk

Early milk (median 45 ml, range 17.5–52 ml,  $n = 22$ ) and mature milk (median 51.5 ml, range 12–104 ml,  $n = 61$ ) were compared for exosomal content and phenotype. We found a lower concentration of exosomal protein (Fig. 1A) and a lower number of vesicles (Fig. 1B) in mature milk compared with early milk. Next, we analyzed the phenotype of exosomes from early milk and mature milk coated on anti-MHC-class II dynabeads. Representative histograms of the markers are shown in Fig. 1C–H. Exosomes in early milk contained significantly higher levels of HLA-DR (Fig. 1E) and expressed lower amount of HLA-ABC (Fig. 1F) compared with mature milk exosomes. No significant differences were detected in the expression of the tetraspanins CD63, CD81, the scavenger receptor CD36 or mucin-1 (MUC1) (Fig. 1C–D, and G–H). The presence of CD36, CD63, and MUC1 was confirmed on sucrose density-purified early milk-vesicles in the density range 1.12 and 1.18 g/ml (Fig. 1I), indicating their exosome origin (12).

### Different populations of exosomes are present in mature breast milk

Phenotyping of exosomes by bead-based flow cytometry implies analyzing a selection of exosomes using antibody-coated beads with the disadvantage of excluding exosomes lacking or having too low expression of the selected molecule. Thus, to obtain a broader characterization of the exosome population in mature milk, we used anti-MHC-class II dynabeads (enriching for exosomes with antigen-presenting potential) and anti-CD63 latexbeads (enriching for exosomes originating from a wide range of cells (20–22)) (Fig. 2A–B, respectively). Representative histograms of the molecules analyzed are shown (Fig. 2). To be able to compare the exosomes selected on the different beads, we analyzed the CD63/HLA-DR ratio obtained. We found that exosomes bound to anti-CD63 latexbeads had a significantly higher CD63/HLA-DR ratio compared with exosomes bound to anti-MHC-class II beads (Fig. 2C), indicating that we captured different exosome populations on the two beads.

### Differences in MUC1 and CD63 on mature milk exosomes depending on maternal sensitization and/or lifestyle

We then addressed whether the phenotype of the identified subpopulations of exosomes in mature milk was influenced by allergic sensitization or lifestyle of the mother. We therefore subgrouped mothers (Table 1) as sensitized or nonsensitized

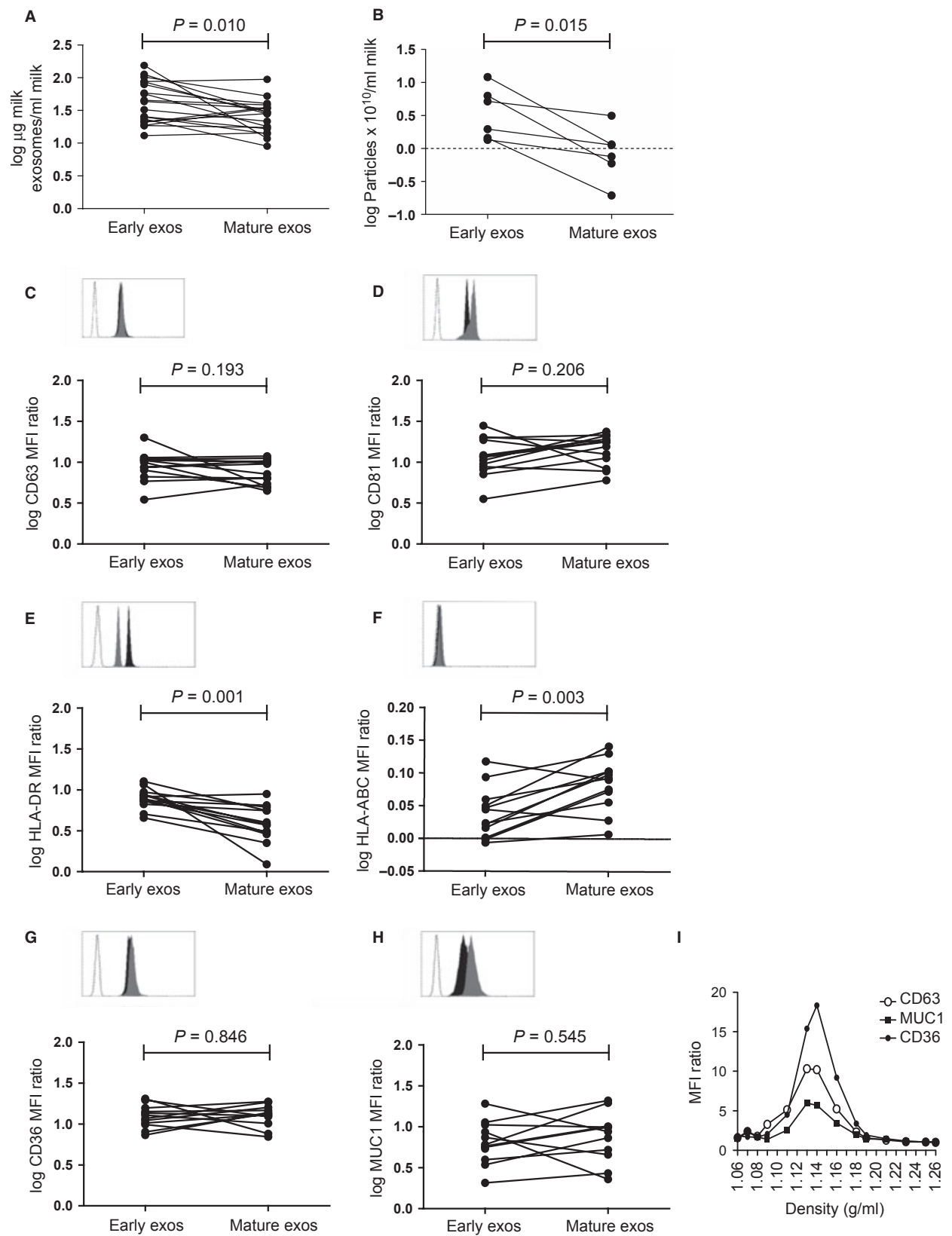


**Figure 1** Phenotypic differences between milk exosomes from early milk and mature milk. (A) Exosomal protein concentration measured by DC protein assay ( $\log \mu\text{g/ml}$  milk) from early milk and mature milk (Early exos and Mature exos;  $n = 19$ ). (B) Early milk and mature milk exosomes ( $n = 6$ ) were analyzed by NTA using NanoSight to determine the number of particles  $\times 10^{10}/\text{ml}$  milk. (C–H) Exosomes from early milk or mature milk were coated on anti-MHC-class II dynabeads and analyzed by flow cytometry for the expression of (C) CD63 ( $n = 13$ ) (D) CD81 ( $n = 13$ ) (E) HLA-DR ( $n = 13$ ) (F) HLA-ABC ( $n = 12$ ) (G) CD36 ( $n = 12$ ), and (H) MUC1

( $n = 11$ ). Representative flow cytometry histograms are shown above each graph to illustrate the abundance of expression on early milk (black) and mature (gray) milk compared with the respective isotype control (dotted line). (I) Early milk exosomes (pool of  $n = 3$ ) were loaded on sucrose density gradient, and fractions were loaded on anti-MHC-class II dynabeads and analyzed for CD36, CD63, and MUC1 expression by flow cytometry. All values were log-transformed ( $\log_{10}$ ) prior statistical analysis and dependent t-test was used.

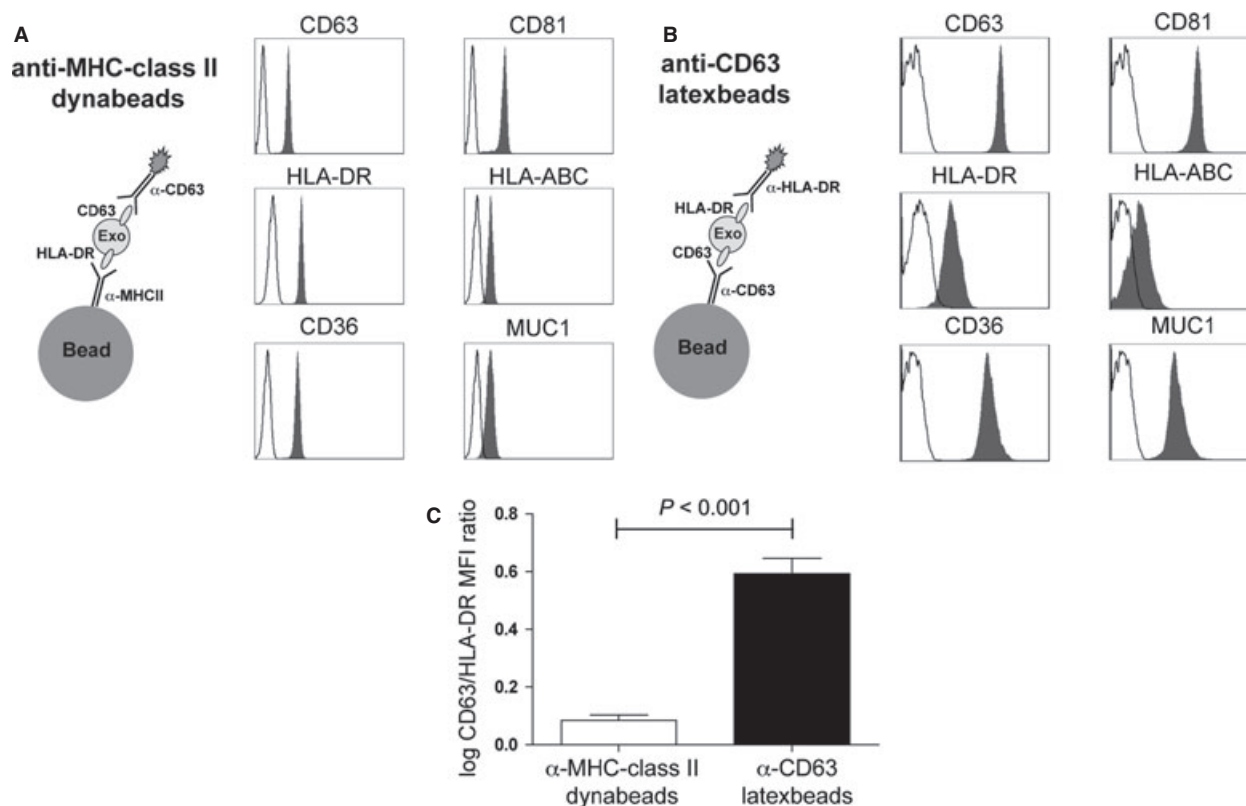


**Figure 2** Different populations of exosomes are present in mature breast milk. (A) Exosomes from mature milk were coated on anti-MHC-class II dynabeads or (B) anti-CD63-coated latexbeads and analyzed by flow cytometry. Representative histograms for the expression level of CD63, CD81, HLA-DR, HLA-ABC, CD36, and

MUC1 on exosomes (black) compared with the respective isotype control (dotted line) is shown. (C) Logarithmic values ( $\log_{10}$ ) of the mean fluorescence intensity (MFI) ratio between CD63/HLA-DR ( $n = 42$ ). Statistical difference was assessed with dependent t-test.

(Fig. 3 A–H) and in a second analysis grouped them as anthroposophic or nonanthroposophic (Fig. 3 I–P). When analyzing exosomes selected onto anti-CD63 latexbeads, we observed a significantly higher MUC1 expression on exosomes from nonsensitized compared with sensitized mothers (Fig. 3H). Interestingly, when relating to lifestyle, exosomes from anthroposophic mothers coated on MHC-class II beads had significantly lower expression of MUC1 compared with nonanthroposophic mothers (Fig. 3L). Furthermore, within the subpopulation of anti-CD63-selected exosomes, we found that anthroposophic mothers had significantly higher levels of CD63 compared with nonanthroposophic mothers (Fig. 3M).

No significant differences in any other marker were detected between the groups (Fig. 3 A–G, I–K, N–P) or for CD36 and CD81 (data not shown).

#### Exosomes in breast milk in relation to sensitization of the child at 2 years of age

Next, we investigated a possible association between the phenotype of exosomes in mature milk and sensitization of the child at 2 years of age (Fig. 4, Table 2). Notably, we observed significantly higher levels of HLA-ABC on exosomes selected for anti-CD63 from mothers whose children developed allergic

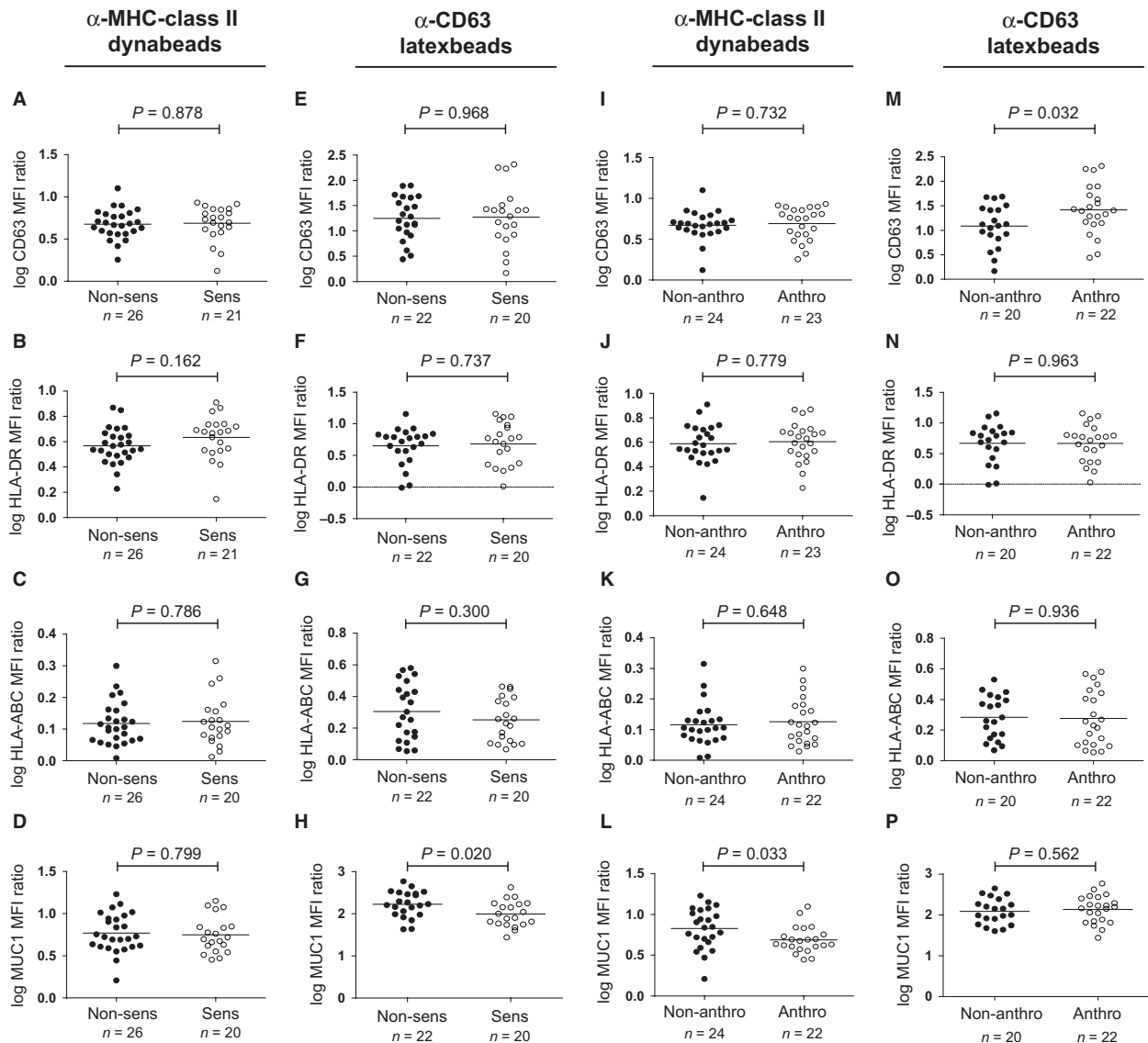


**Figure 3** Exosomes in mature milk in relation to maternal sensitization and lifestyle. Exosomes were coated on either anti-MHC-class II dynabeads (A–D and I–L) or anti-CD63 latexbeads (E–H and M–P) and analyzed by flow cytometry for the expression of CD63 (A, E and I, M), HLA-DR (B, F and J, N), HLA-ABC (C, G and K, O), and MUC1 (D, H and L, P). Flow cytometry data are presented as  $\log_{10}$  mean fluorescence intensity (MFI) values normalized to isotype control.

sensitization compared with mothers to nonsensitized children (Fig. 4C). No significant differences in CD63, HLA-DR, and MUC1 (Fig. 4 A, B, D) or CD36 and CD81 (data not shown) were seen, nor on the subpopulations of exosomes coated on anti-MHC-class II beads (data not shown).

## Discussion

In this study, we discovered phenotypically different subpopulations of exosomes in human breast milk related to sensi-

trol, and the phenotype of exosomes was assessed according to sensitization (A–H, nonsensitized or sensitized) or lifestyle (I–P, anthroposophic or nonanthroposophic) of the mother. Horizontal lines indicate median values, and the dotted lines (F, M) indicate background staining using isotype matched control. ANOVA was used to address statistical difference, and *P*-values are indicated after correcting for lifestyle (A–H) or for maternal sensitization (I–P).

zation or lifestyle of the mother. Moreover, we found an association between the immunological composition of exosomes in mature milk and sensitization of the child at 2 years of age.

We here confirmed our previous finding that the expression of HLA-DR on exosomes decreases in mature milk compared with early milk (12) with a higher number of individuals and a demographically different subject group. Furthermore, we report the novel finding of an increase in HLA-ABC on HLA-DR enriched exosomes in mature milk

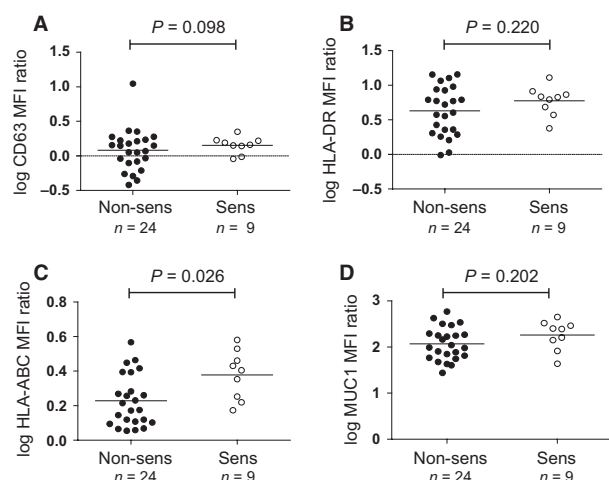


**Figure 4** Exosomes in mature milk in relation to allergic sensitization of the child at 2 years of age. Exosomes were coated on anti-CD63 latexbeads and analyzed by flow cytometry for the expression of CD63 (A), HLA-DR (B), HLA-ABC (C), and MUC1 (D). Log<sub>10</sub> values of the mean fluorescence intensity (MFI) were assessed according to the sensitization state of the child at 2 years of age (nonsensitized, sensitized). Horizontal lines indicate median values, and the dotted lines in (A,B) indicate background staining using isotype matched control. Logistic regression was used and *P*-values are indicated after correcting for maternal sensitization and lifestyle.

compared with early milk. We speculate that the inverse relation of HLA-DR and HLA-ABC on milk exosomes, as well as the finding of a lower number of vesicles in mature milk compared with early milk, is likely reflecting changes in cell populations and/or cellular phenotype in breast milk between early milk and mature milk (5).

The approach of using two different antibody-coated beads for exosome-characterization allowed us to identify different subpopulations of exosomes in mature breast milk enriched for either CD63 or HLA-DR expression. Nevertheless, we need to stress the point that we are likely analyzing overlapping subpopulations rather than two phenotypically distinct populations. The high expression of HLA-DR, MUC1, and CD36 on both subpopulations of milk exosomes examined suggests a mammary epithelial origin (23–25). However, these molecules are also expressed on immunological cells; hence, a portion might originate from immune cells. The selection of exosomes expressing CD63, a widely expressed tetraspanin on exosomes (26), probably leads to the isolation of exosomes originating from various cell types, whereas HLA-DR enriched exosomes could either derive from the mammary epithelium or APC. MUC1 expression was significantly altered on mature milk exosomes, in relation to both sensitization and lifestyle (Fig. 3H and L). Secreted MUC1 from human milk has been shown to adhere to certain pathogenic bacteria (27) and to protect against intestinal viral infection *in vivo* (28). Thus, MUC1-expressing exosomes could potentially function as a decoy

**Table 2** Demographic data on the children at 2 years of age

| Characteristics                         | Sensitized<br>N = 10 | Nonsensitized<br>N = 27 | <i>P</i> -value |
|-----------------------------------------|----------------------|-------------------------|-----------------|
| Parents                                 |                      |                         |                 |
| Mothers sensitized                      | 3/10 (30.0)          | 14/27 (51.8)            | 0.288           |
| Fathers sensitized                      | 1/10 (10.0)          | 8/27 (29.6)             | 0.393           |
| Anthroposophic                          | 3/10 (30.0)          | 13/27 (48.1)            | 0.461           |
| Nonanthroposophic                       | 7/10 (70.0)          | 14/27 (51.9)            |                 |
| Age of mother at birth of child (years) | 32.2 ± 6.0           | 32.3 ± 5.2              | 0.978           |
| Delivery                                |                      |                         |                 |
| Home delivery                           | 1/10 (10.0)          | 6/27 (22.2)             | 0.647           |
| Acute cesarean section                  | 1/10 (10.0)          | 1/27 (3.7)              | 0.255           |
| Planned cesarean section                | 0/10 (0.0)           | 5/27 (18.5)             |                 |
| Breast-feeding                          |                      |                         |                 |
| At 6 month of age                       |                      |                         |                 |
| Exclusive                               | 1/10 (10.0)          | 7/27 (25.9)             | 0.428           |
| Partly                                  | 8/10 (80.0)          | 19/27 (70.4)            |                 |
| Not breast-fed                          | 1/10 (10.0)          | 1/27 (3.7)              |                 |
| At 12 month of age                      |                      |                         |                 |
| Exclusive                               | 0/10 (0.0)           | 1/27 (3.7)              | 0.032           |
| Partly                                  | 1/10 (10.0)          | 14/27 (51.8)            |                 |
| Not breast-fed                          | 9/10 (90.0)          | 12/27 (44.4)            |                 |

Continuous variables presented with mean and SD and tested using independent *t*-test.

Categorical variables presented with n/N and % and tested using Fisher's exact test.

receptor for capturing pathogens and protect the child from early infections. It is tempting to speculate that children breast-fed by anthroposophic mothers will receive less MUC1 on exosomes through breast milk which might render them more susceptible to infections and less likely to develop allergies as a consequence. However, we could not detect an association between the MUC1 expression on the exosomes isolated from mature milk and allergen sensitization of the child at 2 years of age. This could partially be explained by the low number of children who had developed allergen-specific IgE levels  $\geq 0.35$  kUA/l in our study (Fig. 4D), and with only three sensitized children in the anthroposophic group (Table 2). Therefore, the role of MUC1 as a decoy receptor for pathogens and/or commensals in relation to allergen sensitization of the child needs to be further investigated. We found that CD63 expression is significantly increased on CD63-enriched exosomes from anthroposophic compared with nonanthroposophic mothers, indicating either an increase in levels of CD63-expressing exosomes or a specific enrichment in CD63 molecules on exosomes. Whether this has a biological function, for example recruiting leukocytes in the gastrointestinal tract of the infant, remains to be investigated (29). Due to limitations in the number of mothers donating early milk that fulfilled the classification requirements to be included in this study, we were unable to make a similar phenotypic analysis on exosomes in early milk. As the early milk is richer in immunological components compared with mature milk (30), it

might be possible that differences in exosomal profile during early lactation might better relate to allergic outcome of the child. Further studies are required to elucidate these questions.

Our previous studies demonstrating the ability of milk exosomes to induce the expression of Foxp3 and inhibition of cytokine production in PBMC *in vitro* (12), together with additional studies showing that immune-related miRNA are enriched in breast milk exosomes, suggest a potential function of exosomes in cell–cell communication from mother to child (15). Due to the permeable nature of the infant gut (31), where cells from the milk can penetrate (shown *in vivo* in animal models) (32), as well as the relatively alkaline gastric pH and low peptic activity in newborns (33, 34), it is likely that exosomes from milk might play a role in the development of the child's immune system. Interestingly, we here found that children sensitized at 2 years of age had been breast-fed by mothers having significantly higher levels of HLA-ABC expression on mature milk exosomes compared with those whose children remained nonsensitized. Of note, we found no difference in the proportion of sensitized children between anthroposophic and nonanthroposophic families (Table 2,  $P = 0.461$ ). However, information regarding duration of breast-feeding showed that 90% of the sensitized children were no longer breast-fed at 12 month of age compared with 44.4% of the nonsensitized (Table 2), with a potential impact on allergy development/prevention of the child as a consequence. Furthermore, we need to keep in mind that the absolute number of sensitized children was low which could lead to bias. It is not obvious how higher levels of HLA-ABC on breast milk exosomes would be implicated in allergy development. Future larger studies are required to address whether differences in the exosomal phenotype might have a role in allergy development of the child.

It has been shown that several lifestyle factors during the pre- and neonatal period are associated with allergy development and differ between anthroposophic and nonanthroposophic families (4). However, further studies are needed to identify the protective factors with regard to allergy development in the anthroposophic lifestyle. Recently, studies on the ALADDIN cohort have shown that children with an anthroposophic lifestyle have reduced levels of cortisol in saliva (35) and that increased levels of cortisol in infancy are associated to allergic sensitization during the first 2 years of life (36),

suggesting that stress early in life may be of importance for the development of allergy in children.

In conclusion, we show that exosomes, an immunological component in milk, differ between mothers with respect to allergic sensitization and lifestyle, suggesting a potential influence on the immune system of the newborn child as a consequence.

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### Conflicts of interest

The authors declare no conflicts of interest.

### Author contributions

FS, JA, and AS participated in the design and coordination of the ALADDIN study. FS examined all children. PTP and SG were involved in the selection of the collected samples. SG, PTP, CG, CA, and SMJ designed and PTP, CG, CA, and SMJ performed the experiments. PTP, CG, AS, and SG analyzed the data and wrote the manuscript. All authors participated in revising the manuscript and approved the final version.

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