Influence of breast- versus formula-feeding on lymphocyte subsets in infants at risk for celiac disease. The PROFICEL study.

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Abstract

Purpose: In addition to genetic risk, environmental factors might influence celiac disease (CD) development. We sought to assess the effect of the interaction between milk-feeding practices and the HLA-DQ genotype, on peripheral lymphocyte subsets and their activation markers in infants at familial risk for CD.

Methods: 170 newborns were classified in 3 different genetic risk groups (high risk, HR; intermediate risk, IR; and low risk, LR) after DQB1 and DQA1 typing. Lymphocyte subsets were studied at the age of 4 months by flow cytometry analysis.

Results: 79 infants were receiving exclusive breast-feeding (BF), and 91 partial breast-feeding or formula-feeding (FF). Regarding genetic risk, 40 infants were classified in HR group, 75 in IR group and 55 in LR group. Two-way ANOVA did not show significant interactions between the type of milk-feeding and genetic risk group on the lymphocyte subsets analysed. One-way ANOVA for milk-feeding practice alone showed that the percentage of CD4+CD25+ cells was significantly higher in BF group than in FF group (BF, 10.92 ± 2.71 ; FF, 9.94 ± 2.96 ; $p=0.026$), and absolute counts of CD4+CD38+ cells were significantly higher in FF group than in BF group (FF, 2881.23 ± 973.48 ; BF, 2557.95 ± 977.06 ; $p=0.038$). One-way ANOVA for genetic risk alone showed that absolute counts of NK cells were significantly higher in IR group than HR and LR groups (IR, 539.24 ± 340.63 ; HR, 405.01 ± 239.53 ; LR, 419.86 ± 262.85 ; $p=0.028$).

Conclusion: Lymphocyte subset profiles in the early stages of life could be modulated by milk-feeding practices and genetic risk separately. Breast-feeding might have a positive immunomodulatory effect on lymphocyte subsets in infants at risk for CD.

Key words: Lymphocyte subsets, celiac disease, infants, HLA genotype, breast-feeding, formula-feeding.

List of abbreviations:

CD: celiac disease

PCR-SPP: polymerase chain reaction-sequence specific primers

HLA: Human leukocyte antigen

Treg: regulatory T cells

NK: natural killer

LR: low genetic risk

IR: intermediate genetic risk

HR: high genetic risk

BF: breast-feeding

FF: formula/mixture-feeding

Introduction

Celiac disease (CD) is an immune-mediated enteropathy triggered by the ingestion of gluten in genetically susceptible individuals. It is generally accepted that CD is a T-cell mediated disease, in which gliadin derived peptides activate lamina propria infiltrating T lymphocytes. The subsequent release of pro-inflammatory cytokines, in particular γ -interferon, leads to a profound tissue remodelling. However, an innate immune response also seems to be involved in the disease development [1]. This is a complex disorder, with environmental and genetic factors contributing to its aetiology. The major genetic risk factor in CD is represented by HLA-DQ genes. Several studies have documented that the HLA-DQA1*05 and DQB1*02 alleles, encoding for particular DQ2 molecules, confer high susceptibility to CD. This heterodimer can be encoded both in *cis* or *trans*. The susceptibility to CD is increased in homozygous subjects with a *cis* haplotype or possessing a second HLA-DQB1*02 allele [2-4]. In Europe, approximately 90% of patients have these genetic markers whereas most of the remaining cases carry the HLA-DQA1*03 and DQB1*0302 alleles coding for DQ8 molecules [3,5]. Gluten is the main environmental factor responsible for the signs and symptoms of the disease. Moreover, the interplay between other environmental elements and the genetic background is also thought to play a role in the disease risk, including the type of milk-feeding, incidence of infections and intestinal dysbiosis [6].

The effect of environmental factors on future disease risk is relevant at the early stages of life when the immature neonate's gut undergoes the process of microbiota establishment and the immune system acquires full competence and tolerance to non-harmful antigens [7]. A particular feature of the neonatal immune system is the presence of a wide pool of naïve T cells waiting to participate in primary immune responses. Intestinal antigen exposure during neonatal life influences appropriate adult immune responses. T cell differentiation occurs within the neonatal human intestine, and the T cell receptor (TCR) repertoire of these developing immature T cells is likely to be influenced by luminal antigens [8]. Oral ingestion of food and environmental bacteria is a major route of antigenic exposure. After birth, lactation supports immunological defences through a number of molecules with antimicrobial activity and immunologically active components present in breast milk [9,10]. Thus, breast-feeding is known to confer protection against infectious diseases in the short term and there is also some evidence of lower prevalence of inflammatory bowel diseases, childhood cancers and type I diabetes in breast-fed infants [11].

As early events during extra-uterine life allow the maturation of the immune system, the peripheral lymphocyte subsets also exhibit certain changes. Blood cell analysis shows marked lymphocytosis at birth and at later stages of life compared to adulthood. An increase in T and B lymphocytes occurs during the first weeks of life while NK cells decline sharply directly after birth [12,13]. A high CD4/CD8 ratio in babies up to two years of age has been found compared to the values observed in normal adults, and this is mainly due to the large and expanding pool of naïve T CD4+ cells present during the first year of life [14,15]. The ectoenzyme CD38 is expressed in almost all T and B cells [12] and decreases towards the adult life [15,16]. Although breast-feeding is likely to contribute to these changes, only a limited number

of studies have addressed the influence of milk-feeding practices on peripheral lymphocyte subsets or the immunocompetence of lactating children [17-20] and these findings are not consistent among them.

Taking in consideration how important the maturation of the immune system can be as a predisposing or protective factor in future disease development, we planned to assess the effect of milk-feeding practices on the levels of lymphocyte subsets and their activation markers in infants at risk for CD. Since genetic background is a key issue for disease development in this population, we also aimed to find out possible interactions between type of milk-feeding and the HLA genotype on the lymphocyte subsets of these infants.

Materials and methods

Subjects

This study was conducted in 4 month-old infants who are first-degree relatives of CD patients (at least one parent or sibling affected with CD). A total of 170 infants born between October 2006 and the end of 2010 were recruited in 6 hospitals geographically distributed throughout Spain. The 170 infants were divided according to milk-feeding practices into two groups: breast-fed (BF) infants (N=79), including those which had been exclusively breast-fed until the age of 4 months, and formula-fed (FF) infants (N=91), including babies receiving milk formula, either alone or in alternation with breast milk at the moment of analysis. Questions about delivery, infections, vaccinations, feeding practices, clinical symptoms, and medical treatments were answered by the parents at the infant's age of 1 and 4 months in clinical visits with the paediatric gastroenterologist. To facilitate the collection of this information, a diary was given to the mothers upon recruitment (before delivery). The study was approved by the local Ethic Committees and written informed consent was obtained from the parents of children included in the study.

This cohort of infants was analysed for the HLA class II DQA1 and DQB1 genotypes. The infants were classified in three main risk groups, according to their DQ haplotype, loosely based on the criterion of Bourgey et al. and considering the HLA distribution of the Eastern Spanish population [21]. We established the following risk groups: The first one included those individuals carrying the DQ2 haplotype, both in *cis* (DQA1*0501-DQB1*0201 in homozygosis) or *trans* (DQA1*0201-DQB1*0202 with DQA1*0505-DQB1*0301 in heterozygosis) conformation. The second group included those subjects carrying the DQ2 along with any other haplotype, as well as subjects carrying the DQ8 haplotype (DQA1*0301-DQB1*0302) in homozygosis. The third group included those individuals with other common genotypes not associated with CD. Probabilities are approximated and have been estimated taking into account that in most cases the genotype of the celiac relative of each new-born was not considered. 40 infants were in the first risk group (high risk; HR) with the highest probabilities of developing CD (28-24%) [4]. In the second group, with a probability between 7-8%, 75 of the infants were grouped (intermediate risk; IR), and the remaining 55 were in the third group (low risk; LR), with less than 1% risk of developing CD.

DNA isolation

DNA was extracted from buccal mucosa cells by scraping the inner side of the children's cheek with sterile swabs (Copan innovation, Sarstedt, Germany). The cotton was cut and incubated in DLB buffer (100 mM Tris HCL pH 7.4, 10 mM EDTA pH 8, 10 mM NaCl per litre of distilled water); SDS 10% and 10 μ L proteinase K (20 mg/ml) at 65°C for 1 h and then, a standard phenol-chloroform method was carried out. Extracted DNA was stored in TE buffer (10ml Tris HCl pH 8, 200 μ L EDTA 0.5M pH 8 per litre of distilled water) at -20°C after genotyping. The DNA concentration, around 50-100 μ g/mL, was quantified by using the NanoDrop© Spectrophotometer.

HLA-DQ Genotyping

Low-resolution HLA-DQB1 typing was performed by PCR-SSP (Polymerase Chain Reaction-Sequence Specific Primers) analysis. Each PCR reaction was performed on about 60–90 ng of extracted DNA, 0.5 U of BIOTOOLS DNA polymerase (Biotools B&M S.A, Spain), 1 $\times$ PCR Master Mix (DynaL AllSet+™ SSP or Olerup SSPTM) containing nucleotides (200 μ mol L⁻¹ each), PCR buffer (50 mmol L⁻¹ KCl, 1.5 mmol L⁻¹ MgCl₂, 10 mmol L⁻¹ Tris-HCl pH 8.3, 0.001% w/v gelatine), 5% glycerol and 100 μ g mL⁻¹ cresol red, 0.25 μ mol L⁻¹ of each allele- or group-specific primer pair and 0.1 μ mol L⁻¹ of internal positive control primer pair matching a segment of the human growth hormone gene in a final volume of 10 μ L. Detailed PCR protocol: An initial denaturation step at 94 °C for 2 min was followed by 10 two-temperature cycles (94 °C for 10 s and 65 °C for 60 s) and 20 three-temperature cycles (94 °C for 10 s, 61 °C for 50 s and 72 °C for 30 s). Detection of amplified alleles was carried out by 2% agarose gel electrophoresis and ethidium bromide staining. Although the allele DQB1*02 is determinant to be DQ2, HLA-DQA1 alleles were genotyped in a stepwise fashion for a high resolution typing to sharpen the risk classification of each individual.

Blood analyses

At the age of 4 months, peripheral blood samples were drawn from the infants and collected in vacutainer tubes containing K₃EDTA. Immediately after, 1 mL of blood was mixed with an equal volume (1 mL) of preservative solution (Streck Cell Preservative™ CE. Streck, USA) and sent in cool temperature within 2-6 days to our laboratory for a centralised processing and flow cytometry analysis. Blood extractions were always performed previous to the standardised 4 month vaccination in the Spanish vaccination schedule. Complete blood counts and differential counts were performed *in situ* at the corresponding enrolling centres by automated instrumentation. Blood samples were not taken if the infant presented an ongoing infection or had received a vaccination shot in the previous six weeks.

Flow cytometry analysis

Aliquots of blood mixed with the preservative-solution (150 μ L) were incubated for 30 minutes at room temperature and in the dark with 20 μ L of fluorochrome conjugated-monoclonal antibodies specific for CD3+ (CD3-APC), CD4+ (CD4-PerCP-Cy5.5), CD8+ (CD8- PerCP-Cy5.5), CD45RA+ (CD45RA-FITC), CD45RO+ (CD45RO-PE), CD25+ (CD25-FITC), HLA-DR+ (HLA-DR-FITC), CD38+ (CD38-PE), in quadruple immunostainings and 100 μ L aliquots were incubated with the multitests CD3-

FITC/CD16+56-PE/CD45-PerCP-Cy5.5/CD19-APC and CD3-FITC/CD8-PE/CD45-PerCP-Cy5.5/CD4-APC. Fluorochrome-conjugated isotype control immunoglobulins (IgG1 and IgG2a) from mouse were used for each monoclonal antibody to avoid any background fluorescence signal due to nonspecific binding. All the monoclonal antibodies were purchased from Becton Dickinson (Sunnyvale, CA, USA). After incubation, samples were lysed with the BD FACS™ Lysing Solution (Becton Dickinson) following the manufacturer's protocol. The samples were analysed with FACSCalibur Flow Cytometer (four-color, dual-laser, Becton Dickinson). The lympho-gate was defined on the forward and side scatter patterns of lymphocytes. The analysis protocol gated on lymphocytes stained with PerCP and/or APC and the selected population was then analysed with the 2 remaining colours (FITC and PE) to obtain cell percentages expressing the specific antigens. Cell subset counts were obtained by multiplying subset percentages times anchor marker counts, the later resulting by multiplying subset percentage times the absolute lymphocyte count.

Statistics

The normality of the distribution of variables was confirmed through normality graphs and Shapiro-Wilk tests. All variables fitted normal distribution. A univariant analysis of variance was employed with HLA risk and milk-feeding groups as fixed factors to assess the interaction between HLA status and type of milk-feeding. When a significant interaction was found, differences between milk-feeding groups were analysed by T test comparison of means within each risk level, and differences between risk groups by one-way ANOVA within each feeding type level followed by post-hoc analysis. When no interaction was obtained between the two factors, the analyses were done for each factor separately. A *P*-value lower than 0.050 was considered statistically significant.

Results

The demographic characteristics of the infants included in the study are presented in Table 1. All infants were full-term (39.02 ± 1.77 weeks of gestation) and the majority of them were vaginally delivered (118 out of 170). The size and the weight of the infants at the moment of the delivery were within standard ranges and did not differ significantly among the groups.

The percentage and cell counts of the main lymphocyte subsets and subsets expressing activation markers in the whole group of 4-month infants are shown in Table 2. Two-way ANOVA for type of milk-feeding and genetic risk group did not show significant interactions between genetic risk and milk-feeding practices on the lymphocyte subsets analysed.

Two effects of the milk-feeding type alone were found (Table 3). Firstly, one-way ANOVA for milk-feeding practice alone showed that the percentage of CD4+CD25+ cells was significantly higher in BF group than in FF group (BF, 10.92 ± 2.71 ; FF, 9.94 ± 2.96 ; $p=0.026$), and secondly, absolute counts of CD4+CD38+ cells were significantly higher in FF group than in BF group (FF, 2881.23 ± 973.48 ; BF, 2557.95 ± 977.06 ; $p=0.038$). The effects of HLA genotype alone were also analysed (Table 4). One-way

ANOVA for genetic risk alone showed that absolute counts of NK cells were significantly higher in IR group than HR and LR groups (IR, 539.24 ± 340.63 ; HR, 405.01 ± 239.53 ; LR, 419.86 ± 262.85 ; $p=0.028$).

Discussion

In this study, the combined effect of HLA-DQ genotype and milk-feeding practices at 4 month of age on peripheral lymphocyte subsets was studied in a group of infants with at least one first-degree relative suffering from CD. The infants HLA genotype was classified into genetic risk groups in concordance with the last results obtained from the Italian and Spanish population distribution [4,21]. However, taking into account our sample size, the five-group initial classification was reduced to a three-group one. Our analysis did not reveal any interaction between type of feeding and genotype on lymphocyte subsets' profile, but both factors showed an independent effect on specific T cell subsets.

The results obtained for the 20 different lymphocyte subsets measured (median and 10th and 90th percentiles) are in agreement with data published in the literature for 3-6 month old healthy infants [22,15]. However, no reference data have been published so far for this age range in the Spanish population.

A role for breast-feeding as an environmental factor with the capacity to produce immune modulation that might perhaps delay or reduce the risk of developing CD has been suggested by several authors [23-25]. In our population of infants at risk, we have only found a slight modulation of the lymphocyte subset counts by breast-feeding, since most of the subsets did not show differences between breast-fed and formula-fed infants. In the literature, the effects of breast-feeding *versus* formula-feeding on lymphocyte subsets are controversial, but two different studies have found an increased percentage of CD8+ cells in breast-fed infants at 6 and 8 months, compared to formula fed infants [19,20]. In our infants, no differences were found in the CD4+ and CD8+ lymphocytes, however, we found lower CD4+CD38+ counts and higher CD4+CD25+ percentage in breast-fed infants compared to FF infants. CD38+ has a role in T cell activation and differentiation [26] and is constitutively expressed in new-borns decreasing towards the adult life. The fact that lower CD4+ CD38+ counts are found in breast-fed children supports the enhancing effect of breast-feeding on lymphocyte maturation.

Regarding the lower CD4+CD25+ percentage with formula-feeding, this might reflect a lower number of regulatory T cells in these infants. However, since we did not use a specific Treg marker, a major drawback of our study is the impossibility to differentiate within the CD4+CD25+ population what proportion is activated to produce Th1 cytokines and what proportion is Treg to produce IL-10 and TGF- β . The importance of the Treg cells in the susceptibility of atopic disease in children with a familial history of the disease has been pointed out recently [27], even though their role is still not well defined. The modulation exerted by breast-feeding on this population seems relevant, since different animal and human studies have revealed that breast milk induces oral tolerance and prevents asthma and other autoimmune disorders development through an increase of CD4+CD25+Foxp3+ regulatory T cells [28-30]. Several *in vitro* studies showed that specific milk components, like exosomes [31], *lactobacilli* [32],

and lactadherine milk protein [33] activate or increase the number of regulatory T cells (CD4+CD25+Foxp3+). Lactadherine, in addition, has been involved in protection against rotavirus infection [34-37], a pathogen which some investigations have linked to celiac disease development [38]. Overall, our results showing a percentage of T CD4+CD25+ cells significantly higher in BF group than in FF group, and absolute counts of T CD4+CD38+ cells significantly higher in FF group than in BF group, suggest that breast-fed infants could have a more mature immune system than formula fed infants due to the beneficial properties of human milk. This would be an argument in favour of the recommendation to introduce gluten in predisposed infants while they are still breast-fed as a means to possibly lessen the toxicity of gliadin peptides [39]. However, since at the time of our lymphocyte subset analysis, gluten introduction had not occurred yet, it will be interesting to find out if this apparently increased number of regulatory T cell clones is associated with a better response to gliadin after gluten introduction. In addition, there are studies that demonstrate that breast milk contains gliadin peptides [40] and gliadin specific IgA antibodies [40-42] that could be involved in the modulation of the immune response in neonates [40], possibly with a preventive role [42].

Regarding higher absolute counts of NK cells in IR group than HR and LR groups, the possibility exists that this is only a spurious finding, with no biological relevance. However, the association of specific genotypes to a certain marker, a function, or a disease, is usually difficult to explain. In this sense, the concept that genetic factors influence the regulation of lymphocyte subpopulations has been supported by several published studies [43-46]. Therefore, more research is needed to clarify the true meaning of the association between NK cells and HLA-DQ genotype in the population studied.

In conclusion, according to our results, the effect of breast milk on lymphocyte subsets could be beneficial in infants at risk for CD and further studies are necessary to assess the combined effect of milk-feeding practices and gluten introduction practices on that risk.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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Figure 1. Four-color flow cytometry analysis of total blood from a 4-mo-old infant. A) Lymphocytes were gated in the forward scatter (FSC) and side-scatter (SSC) histogram according to the size and complexity of the total white blood cells. B) Helper T cells (CD3+CD4+) were gated and used as the anchor marker to analyze CD4+CD25+ (C) and CD4+CD38+ and CD4+HLA-DR+CD38+ (D).

Table 1. Demographic variables in celiac disease risk infants.

Total subjects (n=170)	
Delivery	
vaginal	118/170
cesarean	52/170
Size (cm)	49.73 ± 2.32
Weight (g)	3270.57 ± 458.64
Weeks of gestation	39.02 ± 1.77
Infants at 4 months of age	
Breast-feeding	Subjects (n)
Exclusive at 4 months	79
Formula feeding	Subjects (n)
Formula before 4 months	91

Table 2. Cell-subset percentages and counts of peripheral blood lymphocytes in 4-month old infants at risk for celiac disease.

Subset	Relative frequencies (%) N=170	Absolute counts (cells/ μ L) N=163
CD45+CD3+ Mature T cells	65 (55-74)	3706 (2563-5780)
CD45+CD4+ Helper T cells	46 (38-56)	2719 (1816-4255)
CD45+CD8+ Cytotoxic T cells	14 (10-22)	831 (520-1439)
CD45+CD3-(CD56+16)+ NK cells	6 (4-12)	373 (189-949)
CD45+CD19+ Mature B cells	23 (15-34)	1291 (755-2206)
CD4+CD45RA+ Naïve T4 cells	87 (77-92)	2264 (1453-3684)
CD8+CD45RA+ Naïve T8 cells	90 (77-96)	722 (447-1217)
CD3+CD45RO+ Pan Memory T cells	12 (7-23)	446 (264-918)
CD3+CD4+CD45RO+ Memory T4 cells	9 (5-14)	321 (190-516)
CD3+CD8+CD45RO+ Memory T8 cells	2 (1-7)	81 (36-277)
CD4+CD25+ Activated T4 cells	10 (7-14)	271 (184-439)
CD8+CD25+ Activated T8 cells	1 (0-4)	11 (4-35)
CD4+HLA-DR Activated T4 cells	3 (2-6)	79 (42-179)
CD4+CD38+ Activated T4 cells	96 (93-98)	2538 (1645-4112)
CD4+HLA-DR+CD38+ Activated T4 cells	2 (1-5)	65 (31-165)
CD8+HLA-DR+ Activated T8 cells	4 (2-16)	40 (14-183)
CD8+CD38+ Activated T8 cells	95 (89-98)	786 (481-1404)
CD8+HLA-DR+CD38+ Activated T8 cells	4 (2-16)	37 (11-166)
CD4+CD28+ Activation primed T4 cells	100 (99-100)	2719 (1787-4245)
CD8+CD28+ Activation primed T8 cells	95 (72-98)	747 (469-1219)
Ratio CD4+/CD8+	3 (2-5)	
Lymphocytes		5805 (3930-8500)

Values are medians (10th and 90th percentiles) of the relative frequencies or absolute counts of cells expressing the indicated markers referred to the lymphocyte population defined by the anchor marker, which appears in first place of the subset name.

Table 3. Lymphocyte subset percentages and counts in celiac disease risk infants classified in groups according to milk-feeding practice.

Lymphocyte subsets		Breast feeding (N=79)	Formula feeding (N=91)	P
CD45+CD3+	%	64±8	65±7	0,626
Mature T cells	cel./μL	3824±1386	4055±1224	0,260
CD45+CD4+	%	46±7	47±7	0,264
Helper T cells	cel./μL	2726±945	2991±989	0,086
CD45+CD8+	%	16±5	15±5	0,262
Cytotoxic T cells	cel./μL	956±513	925±388	0,670
CD45+CD3-(CD56+16)+	%	8±4	7±3	0,085
NK cells	cel./μL	494±346	447±253	0,317
CD45+CD19+	%	23±8	24±7	0,767
Mature B cells	cel./μL	1386±649	1489±666	0,320
CD3+CD45RO+	%	14±7	14±8	0,747
Pan Memory T cells	cel./μL	518±359	583±431	0,307
CD4+CD45RA+	%	86±6	85±9	0,594
Naïve T4 cells	cel./μL	2352±887	2568±908	0,136
CD3+CD4+CD45RO+	%	9±4	10±5	0,423
Memory T4 cells	cel./μL	331±202	392±263	0,109
CD8+CD45RA+	%	88±9	87±9	0,711
Naïve T8 cells	cel./μL	832±456	789±277	0,468
CD3+CD8+CD45RO+	%	3±3	3±4	0,493
Memory T8 cells	cel./μL	125±151	149±209	0,417
CD4+CD25+	%	11±3	10±3	0,026
Activated T4 cells	cel./μL	287±91	289±106	0,889
CD8+CD25+	%	2±1	2±2	0,321
Activated T8 cells	cel./μL	16±16	18±21	0,461
CD4+HLA-DR	%	4±2	4±2	0,919
Activated T4 cells	cel./μL	93±54	103±61	0,315
CD4+CD38+	%	94±11	96±2	0,065
Activated T4 cells	cel./μL	2558±977	2881±973	0,038
CD4+HLA-DR+CD38+	%	3±2	3±2	0,619
Activated T4 cells	cel./μL	76±52	88±56	0,173
CD8+HLA-DR+	%	8±10	7±7	0,637
Activated T8 cells	cel./μL	93±181	72±110	0,375
CD8+CD38+	%	93±11	94±5	0,196
Activated T8 cells	cel./μL	893±503	869±341	0,723
CD8+HLA-DR+CD38+	%	7±10	7±6	0,663
Activated T8 cells	cel./μL	88±175	67±99	0,346
CD4+CD28+	%	100±1	100±1	0,900
Activation primed T4 cells	cel./μL	2718±944	2985±992	0,083
CD8+CD28+	%	90±13	90±13	0,794
Activation primed T8 cells	cel./μL	840±437	815±293	0,672
Ratio CD4+/CD8+	%	3±1	3±1	0,295
Lymphocytes	cel./μL	5985±2036	6308±1876	0,294

Data are Mean±SD. One-way ANOVA (P<0.05).

Table 4. Lymphocyte subset percentages and counts in celiac disease risk infants classified in groups according to genetic risk.

Lymphocyte subsets		High risk (N=40)	Intermediate risk (N=75)	Low risk (N=55)	P
CD45+CD3+	%	66±7	64±8	64±8	0,406
Mature T cells	cel./μL	4008±1419	4149±1391	3621±1017	0,079
CD45+CD4+	%	48±8	46±7	47±7	0,491
Helper T cells	cel./μL	2880±1058	3002±1038	2678±799	0,188
CD45+CD8+	%	15±5	15±5	15±5	0,872
Cytotoxic T cells	cel./μL	942±470	1014±511	834±302	0,086
CD45+CD3-(CD56+16)+	%	6±2	8±4	7±4	0,090
NK cells	cel./μL	405±240	539±341	420±263	0,028
CD45+CD19+	%	23±7	23±8	24±7	0,666
Mature B cells	cel./μL	1365±687	1490±633	1429±678	0,633
CD3+CD45RO+	%	15±9	14±8	13±5	0,462
Pan Memory T cells	cel./μL	603±461	587±430	468±284	0,183
CD4+CD45RA+	%	85±10	85±7	87±5	0,477
Naïve T4 cells	cel./μL	2475±1032	2572±960	2325±697	0,333
CD3+CD4+CD45RO+	%	10±7	9±4	9±4	0,510
Memory T4 cells	cel./μL	395±327	379±202	320±203	0,263
CD8+CD45RA+	%	86±10	87±10	88±7	0,427
Naïve T8 cells	cel./μL	806±393	860±412	738±272	0,200
CD3+CD8+CD45RO+	%	4±3	3±4	3±2	0,418
Memory T8 cells	cel./μL	160±163	155±239	98±79	0,174
CD4+CD25+	%	10±3	11±3	10±3	0,167
Activated T4 cells	cel./μL	269±94	307±92	275±110	0,083
CD8+CD25+	%	2±2	2±1	2±2	0,362
Activated T8 cells	cel./μL	15±23	20±20	13±11	0,084
CD4+HLA-DR	%	3±2	4±2	4±2	0,532
Activated T4 cells	cel./μL	90±58	104±62	96±53	0,486
CD4+CD38+	%	96±2	94±11	96±2	0,332
Activated T4 cells	cel./μL	2777±1049	2829±1080	2567±777	0,328
CD4+HLA-DR+CD38+	%	3±2	3±2	3±1	0,642
Activated T4 cells	cel./μL	77±56	88±59	79±46	0,479
CD8+HLA-DR+	%	7±9	7±9	7±7	0,973
Activated T8 cells	cel./μL	93±195	90±156	62±76	0,488
CD8+CD38+	%	94±5	93±12	95±3	0,376
Activated T8 cells	cel./μL	888±454	941±477	791±289	0,146
CD8+HLA-DR+CD38+	%	7±9	7±9	7±7	0,966
Activated T8 cells	cel./μL	89±1888	84±146	57±72	0,481
CD4+CD28+	%	100±1	100±1	100±1	0,969
Activation primed T4 cells	cel./μL	2873±1058	2996±1038	2671±799	0,187
CD8+CD28+	%	89±13	89±15	92±9	0,349
Activation primed T8 cells	cel./μL	807±310	883±434	762±283	0,177
Ratio CD4+/CD8+	%	3±1	3±1	3±1	0,709
Lymphocytes	cel./μL	6107±2075	6489±2020	5745±1702	0,110

Data are Mean±SD. One-way ANOVA (P<0.05).