Maximally Expressed miRNAs of Milk in Cells, Plasma and Lipid Fraction of Human Milk and Antibodies-Abzymes Catalyzing Their Hydrolysis

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Abstract: Human milk provides neonates with various components that ensure newborns’ growth, including protection from bacterial and viral infections. In neonates, the biological functions of many breast milk components can be very different compared with their functions in the body fluids of healthy adults. Catalytic antibodies-abzymes hydrolyzing peptides, proteins, DNAs, RNAs, and oligosaccharides were detected not only in the blood sera of autoimmune patients but also in human milk. Non-coding microRNAs (18–25 nucleotides) are intra- and extra-cellular molecules of different human fluids. MiRNAs possess many different biological functions, including regulating several hundred genes. Five of them: miR-148a-3p, miR-200c-3p, miR-378a-3p, miR-146b-5p and let-7f-5p were previously found in milk in increased concentrations. Here, we determined number of copies of these miRNAs in 1 mg of analyzed cells, lipid fractions, and plasmas of human milk samples. The relative amount of microRNA decreases in the following order: cells > lipid fraction > plasma. IgGs and slgAs were isolated from milk plasma, and their activity in the hydrolysis of five microRNAs was compared. In general, slgAs demonstrated higher miRNA-hydrolyzing activity than IgGs antibodies. The hydrolysis of five microRNAs by slgAs and IgGs was site-specific. The relative activity of each microRNA hydrolysis was very dependent on the milk preparation. The correlation coefficients between the content of five RNAs in milk plasma and the relative activity of slgAs than IgGs in their hydrolysis strongly depended on individual microRNA and changed from −0.01 to 0.80. Thus, it was shown that milk contains specific antibodies-abzymes hydrolyzing microRNAs specific for human milk.

Keywords: human milk; abzymes; microRNA hydrolysis

1. Introduction

Short non-coding microRNAs (18–25 nucleotides) are intra- and extra-cellular molecules present in various human and animal fluids [1,2]. MicroRNAs have many different biological functions, including the regulation of up to several hundred genes [3,4]. Different changes of microRNAs (microRNA-regulated gene networks) can result in the realignment in the expression of a lot of genes in different cells. It was shown that human milk could contain a few dozens to thousands of various microRNAs [4–19]. MicroRNAs of a mother’s milk have important functions for lactating breasts and infants [5]. The known data strongly support that the milk microRNAs enter the systemic circulation of infants and have tissue-specific developmental and immunoprotective functions [5]. According to the literature data, human milk contains about 1400 mature miRNAs [5–9]. Based on the estimation of the value of the number of real-time polymerase chain reaction (PCR) cycles at which fluorescence exceeds the threshold value, it was concluded that miR-148a-3p, miR-200c-3p, miR-378a-3p, miR-146b-5p and let-7f-5p are contained in...
human milk in increased concentrations [5–9]. It was interesting to quantify and compare the relative content of these five microRNAs.

Antibodies (Abs) against chemically stable analogues of transition states of reactions and natural antibodies-abzymes having catalytic activities are well described in the literature (reviewed in [11–17]). The spontaneous and antigen-stimulated evolution of autoimmune (AI) diseases (ADs) results in the production of abzymes (ABZs) against polysaccharides, lipids, peptides, proteins, RNAs, and DNAs and their complexes [11–17]. In the blood of ADs patients, were found different abzymes directly against antigens mimicking conformations of transition states of chemical reactions. Moreover, secondary anti-idiotypic Abs-abzymes in active sites of different enzymes were also detected, their existence may be explained on the basis of Jerne’s anti-idiotypic network model [18]. The appearance of ABZs in the blood clearly indicates the beginning of AI processes in the mammals [11–17]. By now, abzymes (IgGs, IgMs and IgAs) hydrolyzing RNAs and DNAs [19–23], polysaccharides [24–26], oligopeptides and proteins [27–33] were found in the blood sera of patients with different ADs and some viral diseases [11–17].

Because of the absence of apparent immunization, the existence of any ABZs in people without any immune diseases was considered not possible. For example, au-to-abzymes were not detected in healthy people and patients demonstrating no very severe infringements of the immune status [11–17].

A particular group of healthy people are pregnant and lactating females. Women’s milk contains different Abs (IgGs, IgAs, slgAs and IgMs), and slgAs are the major component (>85–90%) [34,35]. The origin of milk IgGs is still debated; they could be partially produced locally by mammary gland specific cells or partially moved from the circulation system of female blood [34]. IgAs are synthesized by women’s mammary gland B-lymphocytes [35]. IgA antibodies are produced by plasma cells in the mammary stroma, and then they are assembled to dimeric slgAs on the basolateral surface of the epithelium [36]. During lactation, B cells stimulated by antigen in Peyer’s patches switch from IgM production to dimeric IgA and migrate to the mammary gland [37,38].

The immune system of neonates during the first 4–6 months of life is immature: new-borns’ mucous surfaces and respiratory and gastroenterological tracts are still poorly filled with antibodies [39]. Infants begin to produce antibodies in the intestine in the first 3–5 months of life. However, neonates are well protected by antibodies of their mother’s milk (passive immunity), which covers mucous membranes with Abs against bacterial, viral, and other components [39]. Breast milk slgAs are present in high concentration and active during at least 7–8 months after birth and play an important role in maintaining the passive humoral response [40].

Women during pregnancy and after the beginning of the lactation very often demonstrate a sharp exacerbation of AI reactions similar to those in typical autoimmune pathologies, including anti-phospholipid syndrome, systemic lupus erythematosus (SLE), multiple sclerosis (MS), thyroiditis, renal insufficiency, etc. [41–45]. Pregnancy realizes a range of specific changes in the immune system, leading to an increased risk of several diseases exacerbation and adverse maternal and fetal outcomes, including pre eclampsia, fetal loss, and preterm birth [46,47]. SLE is often a disease during pregnancy [48,49] and sometimes leads to harmful situations for the mother and fetus [50]. The incidence of SLE exacerbation usually occurs during pregnancy and within three months after delivery [48,49,51].

There is sometimes a remission of multiple sclerosis in the third trimester of pregnancy, but the disease worsens in the first postpartum period [52]. Autoimmune thyroid reactions are found in approximately 18% of pregnant women [53]. It is important to emphasize that many different ADs could be “triggered” or “activated” in healthy females during their pregnancy and especially after childbirth [25,41–45,54–56]. The pregnancy and the onset of lactation are special periods associated with the changes in the immune system of women [12–17,54,55]. These changes lead to the synthesis of various
autoantibodies and abzymes in the blood and milk of women. The existence of abzymes in the blood and milk indicates the presence of AI reactions in women.

It was proposed earlier that different ADs could be originated from specific defects of hematopoietic stem cells [57]. It was later shown that various ADs development occurs due to specific changes in the differentiation profiles of bone marrow stem cells (HSCs) [58–62].

Spontaneous and accelerated by antigens development of SLE in SLE-prone MRL-lpr/lpr mice [58–60] and multiple sclerosis in EAE-prone C57BL/6 mice [61,62] leads to very similar specific reorganizations of their immune systems, which is bound with a production of abzymes hydrolyzing DNA and proteins.

Very similar changes (as in mice with deep SLE) in the differentiation profiles of HSCs were revealed in lactating mice [58–60]. Such changes in lactating mice are usually temporary and return to normal after 1–3 months. In contrast, there are further changes in the differentiation profiles in mice diseased with SLE during the deepening of the pathology [58–60]. As in mice with deep SLE pathology, the changes in the differentiation profiles in lactating mice lead to the production of abzymes with high catalytic activity.

As shown in a number of works, the abzymes of ADs patients have a large number of different hydrolytic enzymatic activities. Small subfractions of milk polyclonal IgGs and sIgAs also split RNAs, DNAs [54,55], all nucleotides (NMPs, NDPs and NTPs) [56], possess amylase [25], and phosphatase [56] activities. Human milk is a unique source of abzymes with not only hydrolytic but also synthetic functions, including phosphorylation of lipids [63,64], more than 15 different milk proteins [65,66], and polysaccharides [67,68], which were not found in the patients with ADs. Human sIgAs and IgGs possess significantly higher enzymatic activities compared to abzymes of ADs patients [12–17,54–68].

Initially, it was shown that antibodies-abzymes from the blood of patients with multiple sclerosis [69], systemic lupus erythematosus [70], and schizophrenia [71,72] effectively hydrolyze four microRNAs characteristic of these diseases: miR-9-5p, miR-219-2-3p, miR-137 and miR-219a-5p. As noted above, human milk contains many different microRNAs, including these four microRNAs. It has recently been shown that sIgAs and IgGs of human milk effectively hydrolyze four microRNAs characteristic of immune diseases: miR-9-5p, miR-219-2-3p, miR-137 and miR-219a-5p [73,74]. However, these 4 microRNAs are more typical for patients with various ADs. Five microRNAs are most characteristic of human milk: miR-148a-3p, miR-200c-3p, miR-378a-3p, miR-146b-5p and let-7f-5p [5–10].

Taking this into account, in this work, for the first time, a quantitative analysis of these microRNAs and an assessment of the relative activities of sIgAs and IgGs of human milk in the hydrolysis of these five microRNAs were carried out.

2. Results

2.1. Isolation and Quantification of microRNAs

Five miRNAs were isolated and quantitatively analyzed in seven preparations of human milk lipid fraction, cells, and plasmas (number of copies in 1 mg of the analyzed sample (NC/mg)). Data on the content of five different microRNAs in individual preparations of plasmas, cells, and lipid fractions are given in Table 1.
### Table 1. MicroRNA content in 1 mg of individual preparations of milk plasma, cells and lipid fraction and coefficients of correlations (CCs).

| Number of Donor | MicroRNA Content (Number of Copies in 1 mg of Milk Plasma) | Number of Parameter |
|-----------------|-------------------------------------------------------------|---------------------|
|                 | miR-148a-3p | let-7f-5p | miR-146b-5p | miR-200c-3p | miR-378a-3p |
|                 | p1 | p2 | p3 | p4 | p5 |
| 1               | 1.2 x 10^4 | 1.4 x 10^4 | 1.2 x 10^4 | 3.7 x 10^4 | 6.5 x 10^7 |
| 2               | 8.0 x 10^6 | 6.2 x 10^6 | 1.1 x 10^6 | 1.4 x 10^6 | 2.8 x 10^6 |
| 3               | 1.8 x 10^7 | 5.2 x 10^7 | 8.6 x 10^7 | 1.5 x 10^7 | 7.0 x 10^6 |
| 4               | 6.0 x 10^5 | 1.0 x 10^5 | 1.1 x 10^5 | 1.2 x 10^5 | 3.0 x 10^6 |
| 5               | 1.3 x 10^8 | 4.8 x 10^8 | 8.7 x 10^8 | 4.2 x 10^8 | 1.4 x 10^8 |
| 6               | 1.1 x 10^5 | 2.5 x 10^5 | 4.2 x 10^5 | 3.8 x 10^5 | 9.8 x 10^7 |
| 7               | 1.3 x 10^6 | 4.9 x 10^6 | 9.6 x 10^6 | 5.7 x 10^6 | 4.0 x 10^6 |

CCs between parameters: 1-2 (0.20); 1-3 (-0.005); 1-4 (0.67); 1-5 (0.92); 2-3 (0.81); 2-4 (-0.19); 2-5 (-0.07); 3-4 (0.58); 3-5 (0.14); 4-5 (0.66)

| Number of donor | MicroRNA content in cells (number of copies in 1 mg of milk cells) | Number of parameter |
|-----------------|-------------------------------------------------------------|---------------------|
|                 | miR-148a-3p | let-7f-5p | miR-146b-5p | miR-200c-3p | miR-378a-3p |
|                 | p1 | p2 | p3 | p4 | p5 |
| 1               | 8.1 x 10^7 | 5.0 x 10^7 | 1.2 x 10^7 | 9.2 x 10^7 | 1.1 x 10^8 |
| 2               | 1.2 x 10^9 | 4.4 x 10^9 | 9.4 x 10^9 | 5.4 x 10^9 | 2.0 x 10^8 |
| 3               | 5.8 x 10^7 | 4.4 x 10^7 | 1.2 x 10^7 | 2.6 x 10^7 | 1.5 x 10^8 |
| 4               | 9.7 x 10^8 | 8.1 x 10^8 | 2.3 x 10^8 | 4.0 x 10^8 | 1.2 x 10^8 |
| 5               | 3.2 x 10^8 | 5.5 x 10^8 | 2.0 x 10^8 | 5.3 x 10^8 | 4.1 x 10^8 |
| 6               | 8.8 x 10^8 | 4.5 x 10^8 | 1.1 x 10^8 | 2.4 x 10^8 | 1.6 x 10^8 |
| 7               | 4.2 x 10^7 | 3.7 x 10^7 | 1.0 x 10^7 | 2.2 x 10^7 | 2.1 x 10^8 |

CCs between parameters: 1-2 (0.24); 1-3 (0.23); 1-4 (0.28); 1-5 (-0.17); 2-3 (0.99); 2-4 (0.86); 2-5 (-0.37); 3-4 (0.86); 3-5 (0.33); 4-5 (0.04)

| Number of donor | MicroRNA content (number of copies in 1 mg of milk lipid fraction) | Number of parameter |
|-----------------|-------------------------------------------------------------|---------------------|
|                 | miR-148a-3p | let-7f-5p | miR-146b-5p | miR-200c-3p | miR-378a-3p |
|                 | p1 | p2 | p3 | p4 | p5 |
| 1               | 7.2 x 10^7 | 4.1 x 10^7 | 1.6 x 10^7 | 3.1 x 10^7 | 4.1 x 10^7 |
| 2               | 1.3 x 10^9 | 7.2 x 10^9 | 1.6 x 10^9 | 3.4 x 10^9 | 3.7 x 10^7 |
| 3               | 4.0 x 10^8 | 2.4 x 10^8 | 3.6 x 10^8 | 1.6 x 10^8 | 8.3 x 10^7 |
| 4               | 6.2 x 10^8 | 3.1 x 10^8 | 6.4 x 10^8 | 2.6 x 10^8 | 7.1 x 10^7 |
| 5               | 2.0 x 10^8 | 1.0 x 10^8 | 4.6 x 10^8 | 5.9 x 10^8 | 4.9 x 10^7 |
| 6               | 4.1 x 10^8 | 6.6 x 10^8 | 2.0 x 10^8 | 2.1 x 10^8 | 2.3 x 10^7 |
| 7               | 2.6 x 10^7 | 7.4 x 10^7 | 2.3 x 10^7 | 1.9 x 10^7 | 1.6 x 10^7 |

CCs between parameters: 1-2 (0.15); 1-3 (0.40); 1-4 (0.10); 1-5 (0.28); 2-3 (0.38); 2-4 (0.84); 2-5 (-0.32); 3-4 (0.29); 3-5 (0.26); 4-5 (0.48)

CCs reflecting the content of each of the five RNAs (parameters p1–p5) in plasma, cells and lipid fraction

| p1 cells | p1 lipid fraction | p2 cells | p2 lipid fraction |
|----------|-------------------|----------|------------------|
| 1 plasma | 0.57              | 0.49     | 0.18             | 0.30             |
| 1 cells  | -                 | 0.94     | 2 cells          | -                |
| p3 cells | p3 lipid fraction | -        | p4 cells         | p4 lipid fraction |
| 3 plasma | 0.4               | 0.62     | 4 plasma         | -0.22            | 0.003             |
| 3 cells  | -                 | 0.12     | 4 cells          | -                | 0.54              |
| p5 cells | p5 lipid fraction | -        | -                | -                | -                 |
| 5 plasma | 0.25              | 0.46     | -                | -                | -                 |
| 5 cells  | 0.92              | -        | -                | -                | -                 |

* For each value, a mean of three measurements is reported; the error of the determination of values did not exceed 7–10%.
The average content of different microRNAs per 1 mg of three samples (milk lipid fraction, cells, and plasmas) decreased in the following order (NC/mg): let-7i-5p (2.6 × 10[^10]) > miR-146b-5p (2.4 × 10[^10]) > miR-200c-3p (2.6 × 10[^9]) > miR-148a-3p (2.1 × 10[^9]) > miR-378a-3p (8.7 × 10[^8]) (Table 2).

Table 2. Values of relative content of five microRNAs in different fractions of human milk.

| Micro-RNAs     | Cells       | Lipid Fraction | Plasma      |
|----------------|-------------|----------------|-------------|
|                | Average Value (M and IQR) | Diapason * | Average Value (M and IQR) | Diapason | Average Value (M and IQR) |
| miR-148a-3p    | 3.8 × 10[^8]± 1.3 × 10[^8] | 4.2 × 10[^7]–9.7 × 10[^8] | 2.0 × 10[^8]–6.0 × 10[^8] | 2.0 × 10[^8]± 9.0 × 10[^8] | 5.1 × 10[^8]± 2.0 × 10[^8] |
| miR-200c-3p    | 4.0 × 10[^8]± 9.0 × 10[^8] | 1.0 × 10[^7]–9.0 × 10[^8] | 2.1 × 10[^8]–3.4 × 10[^8] | 1.8 × 10[^8]± 4.0 × 10[^8] | 6.0 × 10[^7]–4.0 × 10[^8] |
| miR-378a-3p    | 1.0 × 10[^7]–4.1 × 10[^8] | 6.2 × 10[^8]± 7.3 × 10[^10] | 2.0 × 10[^7]–8.0 × 10[^8] | 4.0 × 10[^8]± 8.0 × 10[^8] | 2.0 × 10[^8]± 1.0 × 10[^8] |
| let-7i-5p      | 4.6 × 10[^10]± 6.0 × 10[^10] | 4.1 × 10[^9]–7.2 × 10[^10] | 4.1 × 10[^9]–7.2 × 10[^10] | 2.5 × 10[^8]± 8.0 × 10[^8] | 2.1 × 10[^8]–1.4 × 10[^9] |
| miR-146b-5p    | 1.2 × 10[^8]± 2.3 × 10[^8] | 4.6 × 10[^8]± 6.0 × 10[^8] | 1.1 × 10[^8]–3.6 × 10[^8] | 2.5 × 10[^8]± 8.0 × 10[^8] | 8.5 × 10[^8]± 1.0 × 10[^9] |
| Average content of all microRNAs | 1.25 × 10[^10]± 1.93 | 1.46 × 10[^10]± 2.4 | 0.18 × 10[^10]± 0.04 | 0.18 × 10[^10] ± 0.04 |

* For each value, a mean of three measurements is reported; the error of the determination of values did not exceed 7–10%. ** The median (M) and interquartile ranges (IQR) were calculated.

The relative average content of all five microRNAs averaged over three types of different samples varied greatly (NC/mg): lipid fraction (1.36 × 10[^10]), cells (1.25 × 10[^9]), and plasmas (0.18 × 10[^10]) (Table 2). In addition, the average relative content of five different microRNAs in the three types of analyzed samples was very different. For example, the content of let-7i-5p decreased in the following order (NC/mg): cells (4.6 × 10[^10]), lipid fraction (2.5 × 10[^10]), and plasma (6.2 × 10[^9]) (Table 2). At the same time, maximal content of miR-146b-5p was observed in lipid fraction (4.6 × 10[^10]NC/mg), then in cells (1.2 × 10[^9]), and plasma (8.5 × 10[^9]). The average content of miR-200c-3p in all three fractions was somewhat comparable (NC/mg): cells (4.0 × 10[^9]), plasma (2.0 × 10[^9]), and lipid fraction (1.8 × 10[^9]) (Table 2). The maximum content in plasma was found for let-7i-5p (6.2 × 10[^9]NC/mg) and miR-200c-3p (2.0 × 10[^9]NC/mg), while the content of other microRNAs was much lower (Table 2). The ranges of change of all estimated parameters, their average values, as well as the values of the medians (M) and interquartile ranges (IQRs) are given in Table 2.

An analysis of the correlation coefficients (CCs) for the content of five types of miRNAs (miR-148a-3p (parameter 1 (p1)), let-7i-5p (p2), miR-146b-5p (p3), miR-200c-3p (p4), and miR-378a-3p (p5)) in individual preparations of plasma, cells, and lipid fractions was carried out. Positive CCs between the five miRNAs in milk plasma varied from 0.14 to 0.92. However, three CCs were negative: p1-p3 (−0.005), p2-p4 (−0.19), and p2-p5 (−0.07) (Table 1). Positive correlation coefficients between the five microRNAs in milk cells varied from 0.04 to 0.99 and there were two negative values p1-p5 (−0.17) and p2-p5 (−0.37) (Table 1). Nine positive CCs in the case of milk lipid fractions varied from 0.1 to 0.84 and the tenth coefficient was negative—p2-p5 (−0.32) (Table 1). Interestingly, in all
three fractions (plasmas, cells and lipid fractions) a negative correlation was observed between parameters p2 (let-7f-5p) and p5 (miR-378a-3p): −0.07—–0.37.

It was interesting how the content of each specific microRNA correlates in three different milk fractions. It turned out that the content of miR-148a-3p (0.49–0.94), let-7f-5p (0.18–0.3), miR-146b-5p (0.12–0.62), and miR-378a-3p (0.25–0.92) in plasma, cells, and lipid fractions are characterized by positive CCs (Table 1). Three CCs characterizing the content of miR-200c-3p in three preparations turned out to be very different: 0.54 (cells-lipid fractions), 0.003 (plasma-lipid fractions) and −0.22 (plasma–cells) (Table 1).

The statistical difference in the content of almost all microRNAs in the cell fraction was significant ($p = 0.001–0.003$), except for 148a-3p-278a-3p and 148a-3p-278a-3p ($p = 0.79$). A similar situation was observed for the lipid fraction ($p = 0.001–0.04$) except for two pairs of parameters: 148a-3p-278a-3p and 146b-5p-let-7f-5p ($p = 0.1–0.96$). The statistical significance of differences in the content of most microRNAs in milk plasma was also high ($p = 0.001–0.01$), except for 146b-5p-200c-3p and 148-3p-378-3p ($p = 0.43–0.87$). Thus, a quantitative analysis of the content of five microRNAs in plasmas, cells, and lipid fractions of human milk was carried out. At the same time, no uniformity in the content of all five microRNAs in each of the three milk fractions (plasmas, cells and lipid fractions) was found. The content of each microRNA in each of the fractions turned out to be specific.

### 2.2. Purification and Characterizing of IgGs and sIgAs

It was shown that IgGs and sIgAs from sera and milk of lactating mothers have several different catalytic activities ([12–17] and refs therein). Methods of purification and characterization, including electrophoretic homogeneity of IgGs and sIgAs preparations used in this study, were described in [73,74]. In addition, the relative activities (RAs) of seven individual IgGs and sIgAs from the milk of healthy lactating mothers hydrolyze four miRNAs (miR-9-5p, miR-137, miR-219-2-3p and miR-219a-5p). Moreover, it was shown that IgGs and sIgAs preparations used in this study do not contain impurities of canonical RNases [73,74]. In this study, we first analyzed the RAs of the same IgGs and sIgAs in the cleavage of five microRNAs: miR-148a-3p, miR-200c-3p, miR-378a-3p, miR-146b-5p and let-7f-5p.

### 2.3. Hydrolysis of microRNAs

The relative activity in the hydrolysis of five microRNAs was analyzed using seven IgG and sIgA preparations isolated from milk plasma as described in [73,74]. Typical patterns of miR-146b-5p and miR-148a-3p splitting by seven milk sIgAs and IgGs are given in Figure 1. Three out of seven IgG preparations are weakly hydrolyzed miR-146b-5p (Figure 1A). The major cleavage sites of miR-146b-5p in the case of IgGs with numbers 3 and 4 are 10C-11U, 9C-10C and 6A-7U, while other IgGs weaker cleave this miRNA in these sites (Figure 1A). Interestingly, 4 out of seven IgG preparations (especially with number 7) effectively hydrolyze miR-146b-5p at 18A-19A and 16U-17C sites. Exactly these 18A-19A and 16U-17C sites of the hydrolysis are the most typical and common for all seven sIgA preparations (Figure 1A). The characteristic sites for IgGs in the case of sIgAs should be attributed to moderate hydrolysis sites.

There are much more major sites for hydrolysis of miR-148a-3p by IgGs and sIgAs than for miR-146b-5p. For most IgGs and sIgAs, the following sites can be classified as major: 12A-13U, 11C-12A, 9G-10A, 6C-7A, 5U-6C and 3U-4U (Figure 1B). At the same time, for some IgGs and sIgAs, individual major hydrolysis sites were observed, which should be classified as average or minor in the case of other antibody preparations.
Figure 1. The patterns of 0.01 mg/mL Flu- miR-146b-5p (A) and Flu- miR-148a-3p (B) hydrolysis by slgAs and IgGs (0.04 mg/mL) from human milk plasmas. The hydrolysis products were detected fluorescence due to the fluorescent residue (Flu) on micro-RNAs 5′-ends after reaction mixture incubation for 1 h. The numbers of slgAs and IgGs, lengths of the splitting products, and the percentage of hydrolysis of two microRNAs by each antibody preparation are indicated in panels (A, B).

This is pronounced especially in the case of slgAs with numbers 4 and 5 that very effectively hydrolyze miR-148a-3p at 14C-15A site (Figure 1B).

Only one common major site of miR-200c-3p hydrolysis for IgGs and slgAs antibodies is 5A-6G site (Figure 2A). Four additional major sites are observed for three slgA preparations with numbers 1, 4, and 5: 11G-12G, 10U-11G, 9A-10U, and 6G-7U. For slgA7, IgG1, IgG3, and IgG5, there is a pronounced average hydrolysis site—14C-15C. Interestingly, slgA7 and IgG2–IgG6, in addition to efficient hydrolysis miR-200c-3p at 5A-6G site, split this microRNA at many other sites with approximately comparable efficiency (Figure 2A).

Figure 2. The patterns of 0.01 mg/mL Flu-miR-200c-3p (A) and Flu-let-7f-5p (B) hydrolysis by slgAs and IgGs (0.04 mg/mL) from human milk plasmas. The hydrolysis products were detected fluo-
rescence due to the fluorescent residue (Flu) on micro-RNAs 5′-ends after reaction mixture incubation for 1 h. The numbers of slgAs and IgGs, lengths of the splitting products, and the percentage of hydrolysis of two micro-RNAs by each antibody preparation are indicated in panels (A,B).

For all IgG preparations, the main major site of let-7f-5p hydrolysis is 7U-8G (Figure 2B). In addition, all IgG preparations effectively hydrolyze this microRNA at two sites: 11A-12G and 10U-11A. Other sites of let-7f-5p hydrolysis in the case of some IgGs should be classified as moderate or minor. For seven slgA preparations, no pronounced common sites of let-7f-5p hydrolysis were observed (Figure 2B). Three IgGs (with numbers 1, 4 and 5) very effectively cleave this RNA at 5U-6A site, while in the case of slgAs, this site is a minor one. 7U-8G site is major for four slgA preparations with numbers 2, 3, 6 and 7 (Figure 2B).

Figure 3 demonstrates the patterns of the hydrolysis of miR-378a-3p by IgG and slgA antibodies.

There are four moderate or minor sites of miR-378a-3p splitting by IgGs and slgAs: 20U-21C, 16C-17A, 15U-16C and 11A-12G. At the same time, 7A-8C, 6G-7A, 5A-6G and 4A-5A sites are major in the case of two slgAs, but they are average or minor for all other antibodies (Figure 3).

Table 3 shows the relative activities of seven individual IgG and slgA preparations and the average values for these preparations in the hydrolysis of five micro-RNAs. Several of the antibody sets did not meet the Gaussian normal distribution. Considering this, for all sets of parameters, we calculated the median (M) and interquartile ranges (IQR) (Table 3).

Figure 3. The patterns of 0.01 mg/mL Flu-miR-378a-3p hydrolysis by slgAs and IgGs (0.04 mg/mL) from human milk plasmas. The hydrolysis products were detected fluorescence due to the fluorescent residue (Flu) on micro-RNAs 5′-ends after reaction mixture incubation for 1 h. The numbers of slgAs and IgGs, lengths of the splitting products, and the percentage of hydrolysis of microRNA by each antibody preparation are indicated in panel.
References between different groups of IgGs and sIgAs were estimated by the Mann–Whitney test. For each value, a mean of three measurements is reported; the error of the determination of values did not exceed *.

Interestingly, in the hydrolysis of miR-148a-3p and miR-200c-3p by sIgAs, on average, was 1.7–1.9 times more active than IgGs (Table 3).

The opposite situation was observed for miR-146b-5p, which was hydrolyzed by IgGs approximately 1.8 times more efficiently than by sIgAs. The average activities of

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**Table 3.** Values of relative activity of seven IgGs and sIgAs in the hydrolysis of five microRNAs, their average values, and coefficient correlations (CCs) and difference between group of parameters (P).

| Number of Parameter | p1 | p2 | p3 | p4 | p5 |
|---------------------|----|----|----|----|----|
| IgG1                | 39.0 * | 62.1 | 69.8 | 72.1 | 99.5 |
| IgG2                | 21.0 | 3.9 | 8.2 | 34.6 | 17.6 |
| IgG3                | 58.2 | 25.2 | 7.2 | 61.5 | 55.6 |
| IgG4                | 22.6 | 15.1 | 8.5 | 63.1 | 97.6 |
| IgG5                | 32.4 | 28.3 | 76.1 | 66.3 | 99.7 |
| IgG6                | 12.7 | 4.7 | 12.1 | 40.21 | 12.3 |
| IgG7                | 21.3 | 82.9 | 17.9 | 76.0 | 42.3 |

| Average values M; (IQR)** | 29.6 ± 15.2 | 31.7 ± 29.9 | 28.5 ± 30.6 | 59.1 ± 15.7 | 60.7 ± 38.6 |
|---------------------------|--------------|--------------|--------------|--------------|--------------|
| Coef. Correl. Difference (P) | 1-2 (0.17); 1-3 (0.21); 1-4 (0.39); 1-5 (0.40); 2-3 (0.37); 2-4 (0.83); 2-5 (0.29); 3-4 (0.48); 3-5 (0.66); 4-5 (0.71) |

**Number of parameter**

| Group number | 6 | 7 | 8 | 9 | 10 |
|--------------|---|---|---|---|----|
| sIgA1        | 98.7 | 99.0 | 32.6 | 99.0 | 54.1 |
| sIgA2        | 13.8 | 4.9 | 17.2 | 26.5 | 24.0 |
| sIgA3        | 33.7 | 52.0 | 43.7 | 62.2 | 40.2 |
| sIgA4        | 32.1 | 77.1 | 12.9 | 83.0 | 48.7 |
| sIgA5        | 67.4 | 94.0 | 9.2 | 98.3 | 39.5 |
| sIgA6        | 81.8 | 4.1 | 21.7 | 24.0 | 13.3 |
| sIgA7        | 66.4 | 40.4 | 9.4 | 70.2 | 14.7 |

| Average values M and (IQR) | 56.3 ± 30.5 | 53.1 ± 39.2 | 21.0 ± 12.9 | 66.2 ± 31.0 | 33.8 ± 15.9 |
|---------------------------|--------------|--------------|--------------|--------------|--------------|
| Coef. Correl. Difference (P) *** | 6-7 (0.3); 6-8 (0.04); 6-9 (0.37); 6-10 (0.06); 7-8 (0.04); 7-9 (0.98); 7-10 (0.84); 8-9 (-0.08); 8-10 (0.36); 9-10 (0.73) |

**CC between IgGs and sIgAs and Difference between IgGs and sIgAs (P)**

| 1-6 (-0.07); 2-7 (-0.41); 3-8 (-0.09); 4-9 (0.87); 5-10 (0.87) |

*For each value, a mean of three measurements is reported; the error of the determination of values did not exceed 7–10%.* **The median (M) and interquartile ranges (IQR) were calculated.*** The differences between different groups of IgGs and sIgAs were estimated by the Mann–Whitney test.
slgAs and IgGs in the hydrolysis of miR-378a-3p and let-7f-5p were comparable (Table 3). The most average increased hydrolysis by slgAs and IgGs was observed for let-7f-5p, and the lowest hydrolysis rate was found for miR-378a-3p (Table 3).

All correlation coefficients (CCs) for seven IgG preparations in the hydrolysis of five microRNAs were positive and varied from 0.17 to 0.83 (Table 3). Some CCs for seven slgA preparations in the hydrolysis of five microRNAs were positive and varied from very low (0.04) to very high (0.98). slgAs showed a weak negative correlation (~0.08) in hydrolysis of miR-378a-3p and let-7f-5p. Interestingly, IgGs and slgAs showed a negative correlation in hydrolysis of miR-148a-3p (~0.07), miR-200c-3p (~0.41), and miR-378a-3p (~0.09). However, a high level of positive correlation (0.87) was observed in the hydrolysis by IgGs and slgAs of two other microRNAs: let-7f-5p and miR-146b-5p (Table 3).

In the case of IgG preparations, a significant difference in the relative activity was observed in the hydrolysis of all five miRNAs ($p = 0.002–0.03$) (Table 3). The statistical difference of slgA preparations in the hydrolysis of five microRNAs was also significant ($p = 0.002–0.03$), except for miR-148a-3p — miR-200c-3p and miR-148a-3p — miR-let-7f-5p ($p = 0.70$), miR-200c-3p — miR-let-7f-5p ($p = 0.41$).

For IgG and slgA preparations, a statistical difference was observed in the hydrolysis of three microRNAs: miR-148a-3p, miR-378a, and miR-146b ($p = 0.002–0.03$), but in the case of two RNAs was not observed: miR-200c-3p and miR-let-7f-5p ($p = 0.52–0.70$).

All antibodies were isolated from milk plasma. Therefore, we compared CCs between concentrations of various microRNAs in plasma (Table 4).

### Table 4. Values of coefficient correlations between concentrations of five micro-RNAs in human milk plasma *

| microRNAs   | miR-200c-3p | miR-378a-3p | let-7f-5p | miR-146b-5p |
|-------------|-------------|-------------|-----------|-------------|
| miR-148a-3p | 0.97        | 0.99        | 0.07      | 0.33        |
| miR-378a-3p | 0.98        | -           | 0.07      | 0.33        |
| let-7f-5p   | 0.007       | 0.07        | -         | 0.79        |
| miR-146b-5p | 0.42        | 0.33        | 0.79      | -           |

* The correlation coefficients between different parameters were calculated.

All CCs were positive, but very different and varied from 0.007 for miR-200c-3p and let-7f-5p to 0.99 in the case of miR-148a-3p and miR-378a-3p (Table 4). It could be expected that the relative activity of antibodies in the hydrolysis of each of the five RNAs would correlate with the relative concentration of these microRNAs in milk plasma. However, this turned out to be far from the case. The CCs of the concentrations of miR-148a-3p (~0.01) and miR-200c-3p (~0.05) in plasma were positive correlation of their hydrolysis by IgGs turned out to be weakly negative. The rest of the CCs were positive: 0.41–0.79 (Table 5). In the case of slgAs, all CCs were positive and varied from 0.03 to 0.8.

### Table 5. Values of coefficient correlations between plasma microRNAs concentrations and relative activities of seven IgGs and slgAs in the hydrolysis of five microRNAs *

| Antibodies | miR-148a-3p | miR-200c-3p | miR-378a-3p | let-7f-5p | miR-146b-5p |
|------------|-------------|-------------|-------------|-----------|-------------|
| IgGs       | -0.01       | -0.05       | 0.79        | 0.41      | 0.53        |
| slgAs      | 0.8         | 0.33        | 0.03        | 0.59      | 0.59        |

* The correlation coefficients between different parameters were calculated.

#### 2.4. Spatial Structures of Five miRNAs

Spatial structures of five miRNAs having minimal free energy were calculated. The relative amount (%) of every product of each microRNA hydrolysis by individual IgGs and slgAs was calculated. Then, using the data of three independent experiments for each IgG and slgA samples, the average percentage of every product corresponding to
seven milk plasma IgG and IgA preparations was calculated. Figures 4–6 show the location of hydrolysis sites in the spatial structures of five microRNAs in the case of IgG and sIgA antibodies. As indicated above and can be seen in Figures 1–3, antibodies from milk plasma of various donors hydrolyze five microRNAs with different efficiencies and, in some cases, at different sites. Taking this into account, Figures 4–6 show averaged data on the efficiency of five microRNAs hydrolysis by seven IgG and sIgA preparations at each of the sites. Pronounced major hydrolysis sites of each microRNA in the case of only some antibody preparations are indicated in brackets.

![Figure 4](image)

**Figure 4.** The average efficiency of Flu-miR-148a-3p (A,B) and Flu-miR-200c-3p (C,D) hydrolysis by seven sIgAs (A,C) and IgGs (B,D) from human milk plasmas in all sites of their cleavage. The average percent of microRNAs and position of separation in different sites of microRNAs hydrolysis by antibodies are shown using spatial models of microRNAs. Values in parentheses indicate the efficiency of microRNA hydrolysis at individual sites by some preparations with an increased specific activity in relation to these sites.

The main sites for more efficient cleavage of miR-148a-3p by IgG and sIgA antibodies are located in the specific loop of this microRNA (Figure 4A,B). In the case of seven sIgAs, the hydrolysis at five sites of the loop is very different. Some sIgA preparations hydrolyze this microRNA at these sites 1.3–3.0 times more efficiently in comparison with the average values for all seven sIgAs (indicated in brackets) (Figure 4A). The hydrolysis efficiency of the miR-148a-3p by seven IgG preparations was more comparable (Figure 4B).

Four of the six hydrolysis sites of miR-200c-3p are also located in the loop of this RNA, but they can be classified as moderate cleavage sites in the case of IgGs (Figure 4D). Several sIgA preparations more efficiently hydrolyze miR-200c-3p at two sites in this loop (Figure 4C). The most efficient hydrolysis of this miR-200c-3p by sIgAs and IgGs occurs at the 5A-6D site outlying from the loop (Figure 4C,D).

The double-stranded loop fragment of miR-378a-3p includes 16 of its 22 nucleotides (Figure 5A,B). In addition, in this case, 8 of 11 hydrolysis sites are located in this specific loop. The relative average percentage of hydrolysis of miR-378a-3p by sIgAs (1.7–5.2%) and IgGs (1.6–4.4%) is relatively low. However, there are three hydrolysis sites of this microRNA in the region from 3G to 6G. In the case of several sIgA preparations, hydrolysis at the 4A-5A site proceeds more efficiently (12%).

Four of the eight hydrolysis sites of let-7f-5p are disposed in its loop, having no double-stranded regions (Figure 5C,D).
Figure 5. The average efficiency of Flu-miR-378a-3p (A,B) and Flu-let-7f-5p (C,D) hydrolysis by seven sIgAs (A,C) and IgGs (B,D) from human milk plasmas in all sites of their cleavage. The average percent of microRNAs and cleavage position in different microRNAs hydrolysis sites by antibodies are shown using spatial models of microRNAs. Values in parentheses indicate the efficiency of microRNA hydrolysis at individual sites by some preparations with an increased specific activity in relation to these sites.

Of the six sites of pronounced hydrolysis, three are also located in a specific loop of miR-146b-5p. Some sIgA preparations hydrolyze these microRNAs at two sites (7U-8G and 10U-11A) more efficiently than other ones (Figure 5D). However, one sIgA preparation hydrolyzes this microRNA most efficiently at 5U-6A site (24.4%). At the same time, the overall sites of maximum hydrolysis of let-7f-5p by sIgAs and IgGs are entirely different: 5U-6A and 7U-8G (Figure 5C,D).

sIgAs and IgGs hydrolyze miR-146b-5p at six sites, three of which are located in the loop. Hydrolysis at these three loop sites is moderate (Figure 6).
Figure 6. The average efficiency of Flu-miR-146b-5p (A,B) hydrolysis by seven slgAs (A) and IgGs (B) from human milk plasmas in all sites of their cleavage. The average percent of microRNAs and cleavage position in different microRNAs hydrolysis sites by antibodies are shown using spatial models of microRNAs. Values in parentheses indicate the efficiency of microRNA hydrolysis at individual sites by some preparations with an increased specific activity in relation to these sites.

The site of maximum hydrolysis of this microRNA by slgAs and IgGs is 9C-10C6 adjacent to the loop (Figure 6). Unlike slgAs (Figure 6A), several IgG more efficiently hydrolyze this microRNA at two sites: 5G-6A and 6A-7U (Figure 6B). Regardless of the absence or presence of double-stranded regions in specific loops, all five microRNAs are mainly hydrolyzed by slgAs and IgGs at the sites of their particular loops. Hydrolysis at some sites of all five microRNAs in the case of slgAs and IgGs is comparable, however, in the case of some sites, significant differences are observed. The most striking differences in the hydrolysis of microRNAs by slgAs and IgGs are observed in the case of a specific hairpin fragment of miR-200c-3p (Figure 4C,D), 4A-5A site of miR-378a-3p (Figure 5A,B), 5U-6A site of let-7f-5p (Figure 5C,D), and 5G-6A and 6A-7U sites of miR-146b-5p.

3. Discussion

MicroRNAs regulate the expression of many genes through association with Argonaute [75–81]. At the same time, miRNAs are susceptible to degradation. Although association with Argonaute protects miRNAs from nucleases, extensive pairing with some unusual RNA targets can trigger miRNA hydrolysis [75,80]. It is believed that the degree of complementarity and the miRNA/target ratio is critical for efficient miRNA hydrolysis [75,77]. MiRNA and target form a duplex with an unpaired flexible linker, which leads to duplex bending and opening of the 3′ end of miRNA for enzymatic attack [77]. According to [76], endogenous RNA (Serpine1) controls the hydrolysis of two miRNAs (miR-30b-5p and miR-30c-5p) in mouse fibroblasts. Targeted miRNA degradation requires ZSWIM8 Cullin-RING E3 ubiquitin ligase [80]. Some studies have found other proteins involved in miRNA degradation, and it is also noted that the unprotected 3′-ends of miRNAs can become available for enzymatic attack by 3′-5′-exonucleases or as yet unidentified other cellular enzymes [75,78]. Thus, destruction occurs in a complex with Argonaute with miRNA at the 3′-end of the molecule.

It was previously shown that Abs from healthy volunteers could not split RNAs and DNAs [12–17]. However, IgGs and IgAs from human milk effectively hydrolyze supercoiled DNAs and polymeric RNAs [12–17,54]. For the first time, the RNase activity of IgGs and slgAs from a mother’s milk in the hydrolysis of four microRNAs was described in [73,74]. It showed that all IgGs and slgAs effectively hydrolyze four microRNAs: miR-9-5p, miR-219-2-3p, miR-137, and miR-219a-5p. Elevated concentrations of these four microRNAs are more characteristic of patients with SLE and MS, and IgGs effectively hydrolyze them from the blood of these patients [69–72]. In this work, a quantitative analysis of the relative content of five microRNAs (miR-148a-3p, miR-200c-3p, miR-378a-3p, let-7f-5p, and miR-146b-5p), which are present in elevated concentrations
in human milk, was carried out for the first time. Interestingly, the average content of the sum of all five microRNAs decreased in the following order (number of copies in 1 mg of sample): lipid fractions \((1.46 \times 10^{10}) \approx \text{cells} (1.25 \times 10^{10}) > \text{plasma} (0.18 \times 10^{10})\) (Table 2).

The statistical difference in almost all microRNAs content in cells, plasmas, and lipid fractions was mainly significant \((p = 0.001–0.04)\), except for some of them \((p > 0.05)\). Positive CCs between the five miRNAs in most preparations of milk plasmas \((0.14 \text{ to } 0.92)\), cells \((0.04 \text{ to } 0.99)\), and lipid fractions varied over a wide range with several exceptions when negative correlations were observed from \(-0.005 \text{ to } -0.37\) (Table 1).

It was interesting how the content of each specific microRNA correlates in three different milk fractions. It turned out that the content of miR-148a-3p \((0.49–0.94)\), let-7f-5p \((0.18–0.3)\), miR-146b-5p \((0.12–0.62)\), and miR-378a-3p \((0.25–0.92)\) in plasmas, cells, and lipid fractions are characterized mainly by positive CCs (Table 1). Three CCs characterizing the content of miR-200c-3p in three types of preparations turned out to be very different: 0.54 (cells-lipid fractions), 0.003 (plasmas-lipid fractions) and -0.22 (plasmas-cells) (Table 1).

IgGs and sIgAs were isolated from milk plasmas, and their relative catalytic activity in the hydrolysis of five microRNAs was estimated (Table 3). It should be noted that the relative activity of IgGs and sIgAs in the hydrolysis of five microRNAs depended very strongly on the milk preparation. For example, the rate of hydrolysis of miR-200c-3p by IgG7 was 21.3 times higher than that for IgG2, while sIgA2 hydrolyzed this microRNA 20.2 times slower than sIgA1. Overall, the efficiency of some microRNAs hydrolysis by seven IgGs and sIgAs did not correspond to the normal Gaussian distribution. CCs of the hydrolysis of five RNAs by the seven IgGs ranged from 0.17 to 0.82, while for 7 sIgAs from -0.08 to 0.98 (Table 3). The CCs between concentrations of five microRNAs in plasmas were also very different and varied from 0.07 to 0.99 (Table 3). CCs between microRNAs concentrations in individual plasmas and RAs of Abs corresponding to these plasmas in the hydrolysis of five microRNAs for IgGs varied from -0.01 to 0.79 and for sIgAs from 0.03 to 0.80 (Table 3). Milk was collected from seven women at about the same time after lactation began. However, in the case of three RNAs, the CCs between the RAs of IgGs and sIgAs were negative (-0.07—-0.41), and for two RNAs, they were highly positive (0.87) (Table 3).

The statistical difference in the content of almost all microRNAs in the cells was significant \((p = 0.001–0.003)\), except for 148a-3p-278a-3p and 148a-3p-278a-3p \((p = 0.79)\). A similar situation was in the case of the lipid fractions \((p = 0.001–0.04)\) except for two pairs of parameters: 148a-3p-278a-3p and 146b-5p-let-7f-5p \((p = 0.1–0.96)\). The differences in the content of most microRNAs in milk plasmas was also high \((p = 0.001–0.01)\), except for 146b-5p-200c-3p and 148-3p-378-3p \((p = 0.43–0.87)\).

The sites of five microRNAs hydrolysis by sIgAs and IgGs are indicated on RNA’s spatial structures (Figures 4–6). Substantially, sIgAs and IgGs hydrolyze five microRNAs at the same sites, mainly located in the loop structures of the substrates (Figures 4–6). The hydrolysis efficiency of five RNAs by sIgAs and IgGs is predominantly comparable. At the same time, several particular of seven sIgA and IgG preparations hydrolyze these microRNAs much more efficiently than others. Hydrolysis of microRNAs occurs after their various bases: A, G, U and C (Figures 4–6). No pronounced specificity of microRNA hydrolysis after a specific base is observed. It is possible that, in principle, the spatial structures of different microRNAs are, to some extent, a more critical factor in determining cleavage sites.

As mentioned above, destruction of microRNAs occurs in a complex with Argo- nauta at the 3’-end of RNAs. It should be assumed that some part of microRNAs in milk can exist in a free form. And these microRNAs can be site-specific hydrolyzed by IgGs not at their 3-terminus.
4. Materials and Methods

4.1. Chemicals and Donors

Most chemicals of high quality used for this study were obtained from Sigma (St. Louis, MO, USA). Protein A-Sepharose, Superdex 200 HR 10/30 column, and Protein G-Sepharose were provided by GE Healthcare (GE Healthcare, New York, NY, USA). FastAP thermosensitive alkaline phosphatase and RNase A were from Fisher Scientific (Pittsburgh, PA, USA). Fluorescein isothiocyanate (FITC) was from Thermo Fisher (Thermo Fisher; MA, New York, USA). FITC-conjugates of oligonucleotides (ONs) were synthesized using the solid phase phosphoramidite method [82]. According to their analysis, all ribo-ONs were homogeneous according to reversed-phase chromatography and electrophoresis in 20% polyacrylamide gel.

RNA was isolated using reagents, buffers, and columns using a special Lira kit (BiolabMix, Russia; https://biolabmix.ru/catalog/nabory-i-reagenty-nk/nabor-dlya-nk/reagent-lira-nabor-lira-dlya-vydeleniya-rnk-dnk-i-belkov/; accessed on 5 January 2017). RNA isolation was carried out according to the instructions of this company.

The milk sampling protocol was confirmed by the Human ethics committee of Novosibirsk State Medical University (Novosibirsk, Russia; number 105-HIV; 07. 2010). The ethics committee supported this study in appliance with Helsinki ethics committee guidelines. All mothers gave written agreement to donate their milk for scientific studies. The mothers have no history of gastrointestinal, respiratory, autoimmune, rheumatologic, cardiovascular, or other system pathologies.

4.2. Purification and Analysis of RNAs

RNA preparations were isolated from different fractions of human milk: cell sediments, lipid fractions, and milk plasmas. A combination of special Lira reagents and buffers and columns (BiolabMix) was used for isolation. 1 mL of a special solution of Lira reagents was added to 100 μL of the sample and incubated for 10 min at room temperature with constant stirring. Then, 200 μL of chloroform was added, and the mixture was incubated for 10 min at room temperature. The resulting mixture was centrifuged for 12 min at 10,000×g at 4 °C. The aqueous upper phase was used for the isolation of RNAs. To RNA-containing aqueous phase, an equal volume of 96% ethanol was added, and this mixture was applied to the silicon membrane of a particular column (BiolabMix). After the column centrifugation in a special tube for 30 s at 10,000×g, at 4 °C, the filtrate realized from the column was removed. Then, to 150 μL of special Lira concentrated buffer WB1, 350 μL of ethanol was added, and 500 μL of the mixture was applied to the column. After centrifugation of the column for 30 s at 10,000×g, at 4 °C, the filtrate was removed. Then, this operation was repeated one more. For complete removal of the buffer, the column was centrifuged again for 3 min at 10,000×g. To remove from the column RNAs, 60 μL of special buffer for RNA elution was applied on the column, which was incubated at room temperature for 5 min, centrifuged for 1 min at 10,000×g, at 4 °C. To remove co-extracted DNA, RNA preparations obtained were incubated for 20 min at 37 °C with DNase I, and for inactivation of DNase I, the solution was heated to 70 °C for 5 min.

4.3. RNA Amplification

RNA amplification was performed according to a standard protocol using special primers on a Bio-Rad CFX Connect device (Hercules, CA, New York, USA).

In the first stage, microRNA reverse transcription was performed using specific Stem-loop primers and a reverse transcription kit including M-MuLV-RH reverse transcriptase (BiolabMix). The reaction mixture containing 2 μL RNA, 1 μL Stem-loop reverse transcription (RT)-primer, 12 μL H2O was heated to 65 °C for 5 min, and then cooled on ice for 2 min. The resulting mixture was centrifuged, 1 μL of M-Mul V and 4 μL of M-Mul V buffer were added. The mixture was incubated in a Bio-Rad CFX Connect cycler.
min at 16 °C followed at 30 °C for 30 cycles, at 42 °C for 30 s, at 50 °C for 1 s and finally at 85 °C for 5 min. Then, reverse transcription products were analyzed by real-time polymerase chain reaction using a fluorescent probe. The reaction mixture (20 μL) contained 10 μL BioMaster HS-qPCR (2x) (BiolabMix), 5.6 μL H₂O, 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM), 1.0 μL fluorescent probe (2.5 μM), and 2 μL reverse transcription product. The reaction mixture was incubated in a Bio-Rad CFX Connect system at 95 °C for 5 min, followed for 45 cycles at 95 °C for 5 s, at 60 °C for 10 s, and 72 °C for 1 min. For reverse transcription of the mRNA of the reference genes, the oligo (dT) primer and the SYBR intercalating dye were used instead of the Stemloop primer.

Primers:
Forward: GTCATCCCTGAGCTGAACGG
Reverse: TTAGGGCAATGCCAGCC

The first step is reverse transcription of microRNAs was performed using oligo(dT) primers and reverse transcription kit containing reverse transcriptase M-MuLV–RH (BiolabMix). The reaction mixture containing 2 μL RNA, 1 μL oligo(dT)-primer, 12 μL H₂O was heated to 65 °C for 5 min, and then cooled on ice for 2 min. After mixture centrifugation, 1 μL of M-MuLV and 4 μL of M-MuLV buffer were added. The mixture was incubated in a Bio-Rad CFX Connect cycler for 30 min at 16 °C, and then for 60 cycles at 30 °C for 30 s, 42 °C for 30 s and at 50 °C for 1 s and finally at 85 °C for 5 min. The reverse transcription products were analyzed by real-time PCR with the SYBR intercalating dye detection. The 20 μL reaction mixture contained: 10 μL HS-qPCR SYBR Blue (2x) (BiolabMix), 6 μL H₂O, 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM), and 2 μL reverse transcription product. The reaction mixture was incubated in a Bio-Rad CFX Connect cycler at 95 °C for 5 min, followed for 45 cycles at 95 °C during 5 s, at 60 °C for 10 s, and at 72 °C for 1 min.

The calibration curves were obtained based on the amplification data of synthetic microRNA used in a concentration from 10⁻⁹ to 10⁻⁷ ng/μL. Using applicator software and calibration curves, the concentrations of each studied microRNA (ng/μL) in different human milk fractions were calculated and recalculated to the number of microRNA copies in 1 mg of the investigated fractions of human milk plasmas, cells, and lipid fractions under study.

4.4. Purification and Analysis of Antibodies

The milk of 7 healthy females residing in the Russia Novosibirsk region (120 mL at one time; 20–35 years old) was collected at 1.0–1.5 weeks after the onset of the lactation. Milk samples were collected using the standard sterile appliances intended for the collection of excess of mother’s milk. During 1–3 h after collection, all samples were cooled to 4 °C, and centrifuged for 20 min at 14 thousand rpm using the Eppendorf centrifuge; cells, lipid phases, and milk plasma were obtained. Immunoglobulins were purified from each milk sample similar to [63–68]. There were no substantial variations in any analyzed parameters of antibodies and abzymes (relative content of Abs and their catalytic activities) within the sampling period of 1–4 weeks after the beginning of lactation [73,74].

To obtain IgGs, the milk plasma was delivered on a column with Protein G-Sepharose equilibrated with buffer A (20 mM Tris-HCl (pH 7.5), 0.15 M NaCl) as in [73,74]. The flow-through fraction containing slgA antibodies was applied on a column with Protein A-Sepharose equilibrated with buffer A. All nonspecifically adsorbed proteins were eluted from Protein G-Sepharose and Protein A-Sepharose first using buffer A up to zero optical density (A280), then with buffer A supplemented with 0.3 M NaCl and 1.0% Triton X-100, and again with buffer A. IgGs and slgAs were specifically eluted from each of sorbents with 0.1 M glycine-HCl (pH 2.6), immediately neutralized using 1.0 M Tris-HCl (pH 8.5), and then dialyzed against 20 mM Tris-HCl (pH 7.5) [73,74].

For additional purification, slgA and IgG preparations (1.0–5.0 mg/mL, 0.3 mL) were incubated in 20 mM glycine-HCl buffer (pH 2.6) supplemented with 0.2 M NaCl at 26 °C for 20–30 min. The Abs were additionally purified using FPLC gel filtration on Superdex...
200 HR according to [63–68]. Collected fractions were immediately neutralized using Tris-HCl buffer (pH 9.0) and then dialyzed against 20 mM Tris-HCl (pH 7.5). For re-folding of Abs after acidic treatment, their RNase activity was measured after 7–14 days of samples stored in this buffer at 4 °C.

4.5. Analysis of microRNA Hydrolysis by Abs

Fluorescently labeled miR-148a-3p (5’-Flu-UCAGUGCACUACAGAACUUGU-3’), let-7i-5p (5’-Flu-UGAGGUAGUAGAUUGAUGU-3’), miR-146b-5p (5’-Flu-UGAGAAACUGUCCAUUCCAUAGGCUG-3’), miR-200c-3p (5’-Flu-UAUAUCUGCCGGUAAUGAUUGA-3’, and miR-378a-3p (5’-Flu-ACUGGACUUGAGUCAGAAGGCG-3’) containing fluorescent residue (fluorescein, Flu) on their 5’-terminus were used. These microRNAs were chosen since they were found in human milk in increased concentrations [5,9].

The reaction mixture (15 μL) contained 50 mM Tris-HCl buffer (pH 7.5); 0.01 mg/mL one of labeled microRNA and 40 μg/mL IgG or sIgA antibodies similar to [73,74]. Final mixtures were incubated for 1 h at 37 °C. The reactions were stopped by the addition of a special denaturing buffer (15–20 μL) containing 8.0 M urea and 0.025% xylene cyanol. The products of all RNAs hydrolysis were analyzed using 20% PAGE and denaturing conditions (0.1 M boric acid, 0.1 Tris, 8.0 M urea and 0.02 M Na₂EDTA, pH 8.3). Each gel was analyzed by Typhoon FLA 9500 laser scanner (GE Healthcare, New York, NY, USA). To get markers of ON length, limited statistical hydrolysis of microRNAs with unspecific alkaline RNase (splitting RNAs with comparable efficiency at all internucleoside bonds) and specific RNase T1 hydrolysis was used. The products after alkaline hydrolysis contained cyclic 3’-monophosphate that possess lower electrophoretic mobility; they give additional bands. Therefore, they were treated with FastAP therinally sensitive alkaline phosphatase. All gels were analyzed using Typhoon FLA 9500 laser scanner (GE Healthcare, New York, NY, USA). The results are reported as a mean ± standard deviation of at least three independent experiments.

4.6. Spatial Model of microRNAs

The spatial models of four microRNAs were generated by Predict a Secondary Structure server, which uses a combination of four algorithms for predicting the secondary structure of RNA similar to [69–71]: calculating a partition function, denoting the structure with minimal energy by http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html (accessed 14 February 2018).

4.7. Statistical Analysis

The relative activities of the IgG and slgA preparations were estimated from a decrease in the fluorescence intensity of the initial microRNA in comparison with that of the control experiment corresponding to incubation of five microRNAs without Abs. The results were given as the mean and the standard deviation of at least two-three independent experiments for each IgG and slgA preparation. Most of the sample sets did not meet the normal Gaussian distribution. To check for a normality of the values distribution law the criterion of Shapiro-Wilk’s W Test was used. The correlation coefficients between different parameters were calculated using the criterion of Shapiro–Wilk’s W Test. The differences between different groups of microRNAs of various groups of IgGs and slgAs sets were estimated by the Mann–Whitney test (Statistica 10; Statistical Package, StatSoft. Inc., USA; http://www.statsoft.com/Products/STATISTICA-Features; StatSoft. Inc., New York, accessed 20 January 2010), the value p < 0.05 was considered statistically significant. The median (M) and interquartile ranges (IQR) are estimated.
5. Conclusions

In summary, we have quantified the relative concentrations of five microRNAs (miR-148a-3p, miR-200c-3p, miR-378a-3p, miR-146b-5p and let-7f-5p) which are found in a mother’s milk in increased concentration. It was shown that human milk polyclonal sIgAs and IgGs possess site-specific microRNAs-hydrolyzing activities. It is shown that each milk preparation is characterized by a specific content of five microRNAs, IgG and sIgA antibodies-enzymes that hydrolyze these microRNAs. The correlation coefficients of the content of five microRNAs in three fractions of milk: cells, plasmas, and lipid fractions and the content of microRNAs in plasmas with relative activity of sIgAs and IgGs in the hydrolysis of these five RNAs vary over a wide range from negative to positive.

Author Contributions: Conceptualization, V.N.B. and G.A.N.; methodology, V.N.B.; formal analysis, V.N.B. and G.A.N.; investigation, I.Y.K. and E.A.E.; resources, G.A.N.; data curation, V.N.B. and G.A.N.; writing—original draft preparation G.A.N.; writing—review and editing, G.A.N.; project administration, G.A.N.; funding acquisition, G.A.N. All authors have read and agreed to the published version of the manuscript.

Funding: This research was performed due to a grant from the Russian Science Foundation, 22-15-00103.

Institutional Review Board Statement: The protocol of blood sampling was confirmed by the local Human ethics committee (Novosibirsk State Medical University, Novosibirsk, Russia; number 105-HIV; 07. 2010). This ethics committee supported this study based on the guidelines of the Helsinki ethics committee. Informed Consent Statement: All MS patients made a written agreement to give blood for scientific investigation.

Informed Consent Statement: The milk sampling protocol was confirmed by the Human ethics committee of Novosibirsk State Medical University (Novosibirsk, Russia; number 105-HIV; 07. 2010). The ethics committee supported this study in appliance with Helsinki ethics committee guidelines. All mothers gave written agreement to donate their milk for scientific studies. The mothers have no history of gastrointestinal, respiratory, autoimmune, rheumatologic, cardiovascular, or other system pathologies.

Data Availability Statement: The data supporting our study results are included in the article.

Conflicts of Interest: The coauthors have no any competing financial interest.

Abbreviations

Abs: antibodies; ABZs, abzymes or catalytic antibodies; ADs, autoimmune diseases; AI, autoimmune; EAE, experimental autoimmune encephalomyelitis; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G; HSC, hematopoietic stem cells; MS, multiple sclerosis; NC/mg, number of copies in 1 mg of the analyzed sample’ PAGE, polyacrylamide gel electrophoresis; PCR, real-time polymerase chain reaction; RA, relative activity; SDS, sodium dodecyl sulfate; sIgA, secretory immunoglobulin A; SLE, systemic lupus erythematosus.

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