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FACOLTÀ DI SCIENZE E TECNOLOGIE

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ANTIVIRAL PROPERTIES OF HUMAN MILK: FOCUS ON RESPIRATORY VIRUSES

Relatore: Prof.ssa Sara Epis
Correlatore: Dott.ssa Lucia Signorini

Tesi di Laurea di:
Francesca Camurri
Matr. 03598A

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SUMMARY

ABSTRACT

Background: Human milk, source of antimicrobial defense for infants, is crucial for the short and long term health and has already demonstrated antiviral activity, due to its matrix of bioactive factors, comprising nonspecific antiviral components and specific antibodies. In particular, human milk confers protection against respiratory infections, drastically reducing morbidity and mortality in newborns, but its live virus neutralizing capacity has been rarely explored *in vitro.*

Aim: The aim of the study was to determine the neutralizing ability of both colostrum and mature human milk against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and human Adenovirus C.1 (HAdV), defining the role of the specific IgG, and the nonspecific molecules, such as the fatty acids and the human milk sialylated oligosaccharides (HMO).

Materials and methods: Milk was collected from two cohorts of mothers, 38 women enrolled at Policlinico IRCCS Cà Granda Ospedale Maggiore Policlinico (POLI) and 36 women enrolled at IRCCS Materno Infantile "Burlo Garofolo" (TS), from 4 to 360 days postpartum. Mothers enrolled in TS were subjected to COVID-19 vaccination a mean of 40 days before the milk collection. Total IgA and SARS-CoV-2 IgG were quantified in the samples by means of ELISA test, while anti SARS-CoV-2 and anti HAdV neutralizing activity (NT_{50}) was evaluated infecting permissive cell lines, Vero E6 and A549, respectively, using the $TCID_{50}$ method. Fatty Acids composition of POLI milk group was defined by means of gas chromatography separation and analyses; HMO antiviral activity was tested *in vitro*, in Calu-3 infected cell model.

Results: IgA were present in 100% of POLI colostrum, POLI mature milk, TS mature milk samples, with a median value of 950000 ng/mL (range 287860-950000 ng/mL, 245180-972050 ng/mL, 271990-998930 ng/mL, respectively). Anti-SARS-CoV-2 IgG were present in 66,6% of POLI colostrum samples, with a median value of 2425 ng/mL (range 1605,75-41451 ng/mL), in 65% of POLI mature milk samples, with a median value of 2063 ng/mL (range 1598,65-29767,45 ng/mL) and in 84.3% of TS mature milk with a median value of 6925 ng/mL (range 1821,75-60140 ng/mL), with a significant difference between POLI and TS mature milk samples (p=0,004). The SARS-CoV-2 median NT_{50} values were as follows: 1:5,42 (range 5,19-41,50) in POLI colostrum samples, 1:2,83 (range 2,24-20,75) in POLI mature milk samples, 1:0 (range

2-19) in TS milk samples. A significant difference was found between median SARS-CoV-2 NT_{50} value of POLI Colostrum and TS Milk groups (p=0,047). No neutralizing activity against HAdV was observed. A strong positive correlation (0,62) between TS milk samples-NT_{50} titers and Anti SARS-CoV-2 IgG, was found. A moderate positive correlation (0,36- 0,30) between SARS-CoV-2 NT_{50} and both Nervonic and Lignoceric FAs was found, while for Palmitoleic acid a moderate negative correlation was found. HMO showed an increasing antiviral activity (20-50%) when used at the concentration 0.002-0.125 ng/mL.

Conclusions: Human milk showed different behaviors against SARS-CoV-2 and HAdV, probably due to the characteristics of the enveloped and naked virions. Although specific anti-SARS-CoV-2 IgG have been observed in almost all the collected samples, their presence significantly correlates with neutralizing activity only in mother subjected to recent vaccination: nonspecific neutralizing activity was probably due to other milk components, such as Nervonic and Lignoceric FAs. Given also the moderate antiviral activity of HMO observed *in vitro*, it is likely that the synergistic effect of all the components of the complex human milk matrix is essential, in order to increase as much as possible the protection of the infants against the respiratory viral infections.

INTRODUCTION

1.1 Human milk physiology

Human milk is the secretory product of the mammary gland that supports the life of the newborn [1]. It comes from the hormonally regulated activities of the mammary epithelial cells and it's the result of different processes, ranging from the development and differentiation of the mammary gland, strictly connected with the state of pregnancy, to psychological aspects, which characterized the relationship between mother and baby[1].

Human milk is produced by the epithelial cells of alveoli (fig.1). Alveoli are the basic secretory units and are clustered in bigger structures: lobules. Different lobules form a lobe. Lactiferous ducts connect each lobe to the nipple: the whole structure has a treelike pattern and it consists in parenchyma tissue. Alveoli are surrounded by myoepithelial cells which play an important role in milk ejection. The other component of the mammary gland is the stroma: connective-tissue, fat tissue, blood vessels, nerves, lymphatics [2,3]

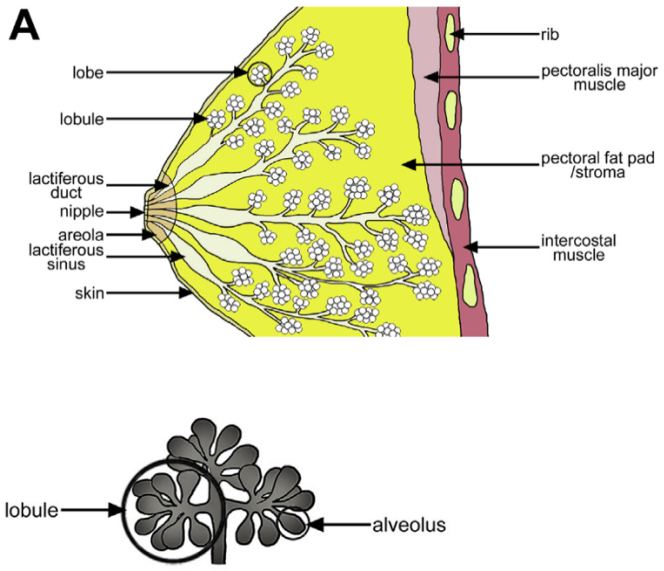


Figure 1 Anatomy and functional organization of the lactating mammary gland. S. Truchet, E. Honvo-Houéto

To understand how milk is secreted it's necessary to consider the development of the mammary gland and its changes in shape, size and function throughout the female' different stages of development [4,5]

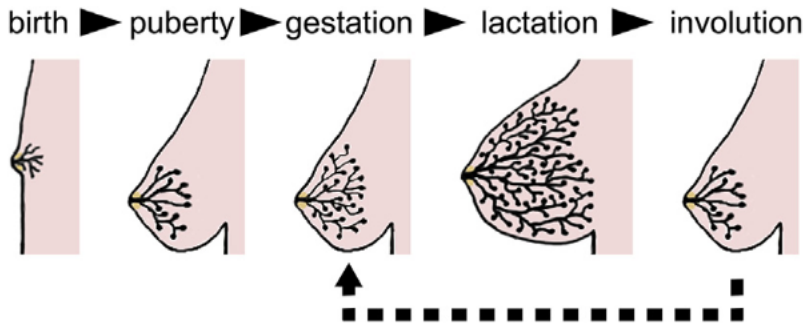


Figure 2 Mammary gland development.Physiology of milk secretion. S. Truchet, Honvo-Houéto.

The first stage of development of the mammary gland takes place during embryogenesis (fig.2). It consists in the elongation, canalization and branching of a first precursor epithelial ectodermal bud localized along the anterior body wall and it consists also in the formation of the fat pad precursor [1]. At birth, in the newborn breast, there is a simple structure of ducts ending in short ductules, surrounded by two layers of epithelial cells, and one of myoepithelial cells. In this phase, a secretory activity can be detected, because of the high maternal hormonal levels, but it regresses within 3-4 weeks [5].

After birth, the mammary gland continues to growth in parallel with the global growth of the organism but the main changes start at puberty. It' s important to underline that variations along changes and developments of the gland occur from woman to woman [5]. At puberty, the increase in estrogen levels stimulates the allometric growth of both the mammary ducts and the adipose tissue [1]. The ducts elongate, branch and divide in more biforcations and apparent main axes and secondary axes. Alveolar buds start to develop at the end of the ducts, being a sort of precursors of the mature terminal organs (fig.3). Infact, different type of lobule-alveolar structures can be distinguished, following the dynamic growth of the mammary gland, from lobule type 1, during puberty, and in nulliparous women breasts, to lobule type 3/4 during pregnancy, and in parous women breasts [5]. First lobulo-alveolar structures are made up 1/2 years after the first menstrual period, and then, with every ovulatory cycle, they grow and regress following the hormonal fluctuations [6].

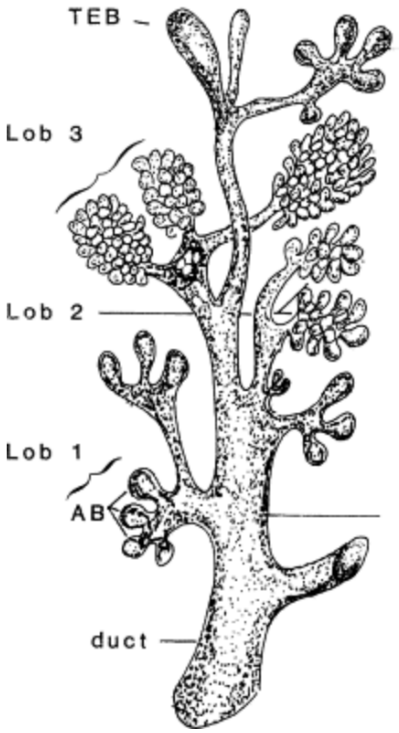


Figure 3. Diagrammatic representation of the lobular structure of the human breast. "Development of the human breast", Russo & Russo 2004.

The secretory activity underlying the breast milk production is achived with alveolar mammary epithelial cells' differentiation, that starts during pregnancy [5]. Two phases can be distinguished: the secretory differentiation and the secretory activation.

During the first half of pregnancy, lobules structures increase in number, size and complexity. The number of alveoli in each lobule increases, epithelial cells of each alveoli go through an active cell division, and become more wide because of cytoplasm enlargement [5]. Ducts elongate, the fat-pad goes through a reduction, extracellular matrix components become more consistent, protecting and supporting mammary epithelial cells [1].

Reproductive hormones and metabolic hormones are involved in the development of the mammary gland, coordinating the reproductive and metabolic state of the organism to the formation and functions of the gland [2]. Alveolar structures' growth during pregnancy is regulate by systemic hormones, such as progesterone, prolactine, growing hormon, glucocorticoids, parathyroid hormone-related protein, and by local factors such as insulin-like growth factor-1 (IGF-1), EGF, and fibroblast growth factor (FGF), which are likely produced by the stromal cells[1]. The actions of each hormone and local factor are not fully understood. The role of progesterone in alveogenesis has

been confirmed by knock out- gene experiments in mice: progesterone receptor knock out mice don't show proliferation of the mammary epithelium. Prolactine binds to prolactine receptor expressed in mammary gland, activating an intracellular signal that ends with the expression of milk protein genes and is able also to stimulate the IGF-1 secretion.

The growing factor contributes to the developing of ducts and probably also to secretory differentation [2].

At the end of the first half of pregnancy, the main tree structure is settled. Branching and lobules' develop continue till parturition and also during lactation: new lobules grow in parallel with milk production. [5].

Secretory differentiation, or lactogenesis 1, begins in the second half of pregnancy. Changing in the expression of biosynthetic enzymes genes and of some milk protein genes such as β-casein, α-lactalbumin, whey acid protein, WDNM1, leads to the differentiation of the secreting units: alveoli start to produce secretory materials, also indicated as colostrum, which accumulates in the lumen [5,7]

Cytoplasmatic lipid droplets accumulate in the differentiating epithelium. [1]

Increasing in lactose and α-lactalbumin blood concentrations are markers of secretory differentiation. [8]

The secretory process remains inhibited by progesterone, till parturition [4]. Unlike other species, in humans, secretory activation (or lactogenesis 2) begins approximately 48 hours after birth, while the actual and consistent production of milk occurs approximately 4-5 days after birth.

Colostrum is the source of support for the baby immediately after birth.

After parturition, the levels of progesterone, estrogen, placental lactogen decrease. Prolactin level generally increases before delivery and remains elevated after delivery. Prolactin, cortisol and insulin stimulate lactogenesis 2. [4]

In the alveolar cells the expression of milk protein genes and biosynthetic enzymes become more homogeneous and several morphological changes occur, such as polarization of organelles, expansion of mitochondria and RER, maturation of the Golgi apparatus, closure of tight-junction complexes that inhibit paracellular transport of nutrients and components, obliging to transcellular transport. [7]

Nutrients and other precursors of milk component reach the mammary epithelium trough blood and lymphatic system and diffuse in the interstitial space.

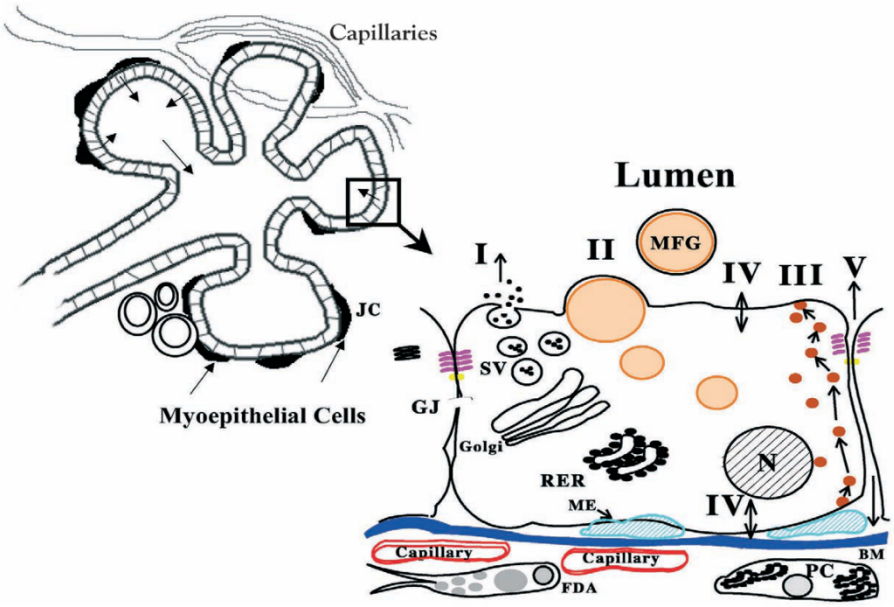


Figure 4 Diagram of mammary alveolus and alveolar epithelial cell showing pathways for milk secretion. "Mammary physiology and milk secretion", McManaman J, Neville M. 1: exocytosis 2: budding process. 3: transcytosis. 4: m transporters activity. 5: no

Depending on type and nature of components and molecules, different transport mechanisms are active both at the basolateral and apical plasma membrane of secretory cells (fig.4). Molecules are taken in at the basolateral membrane and then are secreted from the apical membrane into the lumen of alveoli.

The main transport routes are:

- Membrane transporters activity (4), for ions/ glucose / amino acids / water;
- Transcytosis (3), for Igs/ albumin/ transferrin/ insulin/ prolactine/ estrogen/ cytokines/ lipoprotein lipase;
- Exocytosis (1) for oligosaccharides/ lactose/ milk protein (caseins);
- Budding process (2), for lipid droplets.

The milk secreted is stored in the lumen of alveoli. The baby suckling stimulates a neuroendocrine reflex that consists in the release of oxytocin from the mother posterior pituitary gland. Oxytocin binds to specific receptor expressed on myoepithelial cells stimulating their contraction and the expulsion of milk out of alveoli into the ducts. [6]

1.2 Human milk composition and benefits

Breast milk is divided into colostrum, transitional milk and mature milk, based on the composition' changes that occur in the days and weeks after birth, respectively 5 days for colostrum, from day 5 to day 14 for transitional milk and from day 14 for mature milk[9]. The composition of breast milk reflects the needs of the newborn and its study allows to know which molecules are crucial and supportive for early human growth and development[10] : protective molecules towards potentially pathogenic microorganisms, molecules capable of supporting the development of the immune system, microorganisms that represent a sort of inoculum for the colonization of the still sterile infant gut and undoubtedly, nutrients. In the past, various researches have found positive effects of breastfeeding on children's health and in the prevention of diseases and, subsequently, it has been understood how the benefits of breast milk are actually acquired, i.e. which molecules are involved and how they act [9]. It has been reported how important is studying the network of the molecules in breast milk, trying to verify how they act in synergy with each other and not just singularly[11]. Some components are present in the milk of all mothers without differences, while other components change and fluctuate, based on the mother's diet, the psychophysical state of health, of the mother and the child, on the geographical-environmental location and on the stage of breastfeeding.[10] It is interesting to consider how breast milk is personalized for the child who receives it [11]; and being a complex secretion, many molecules have actions that are not yet completely understood [10].

Human breast milk contains (fig 5):

- Nutrients
- Minerals and vitamins
- Immune factors
- Hormones
- Growth factors
- Microbiome
- microRNAs

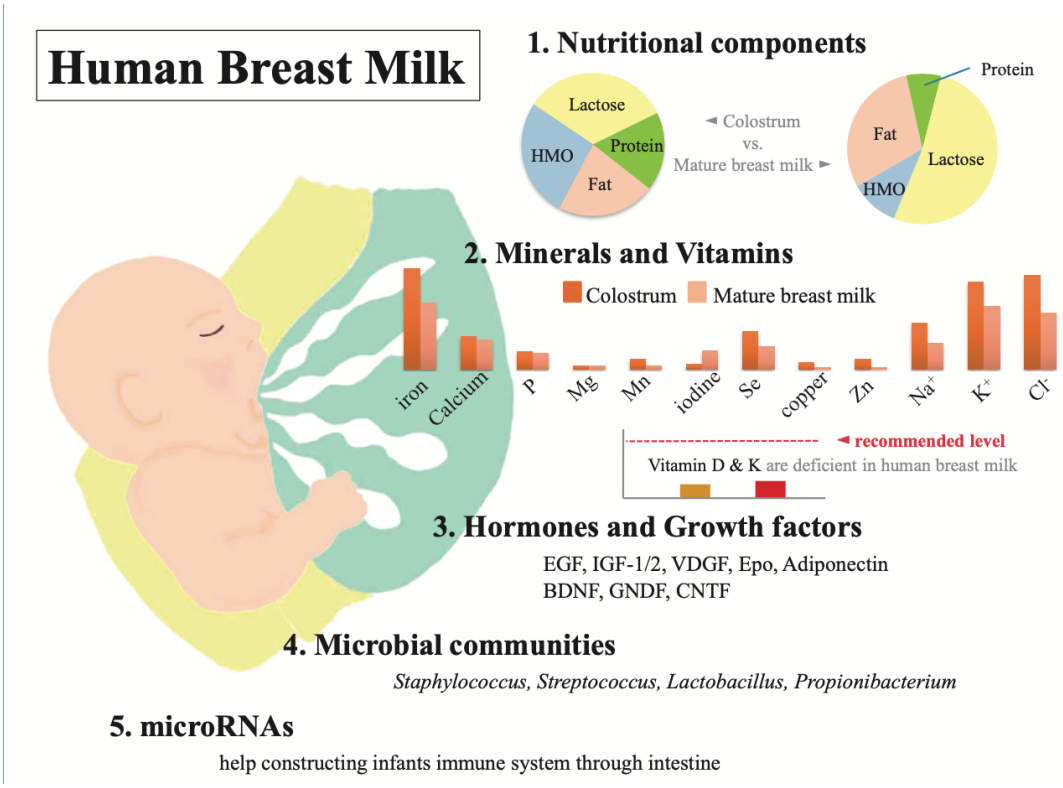


Figure 5. Components of human breast milk. Su Yeong Kim, MD.

Among nutrients, in general, the composition in terms of percentage is:

- 87-88% water - 7% carbohydrates
- 3,8% fat -1% protein -0,2% vitamins, hormones

The composition of colostrum is high in protein and immune components, but less rich in carbohydrates and fats, compared to mature milk (fig.5) [11].

Carbohydrates cover the 40% of the child's nutritional requirement. Among carbohydrates, lactose is the most abundant. On the apical surface of the baby's gut enterocytes, the enzyme lactase digests lactose in glucose and galactose. In human milk, lactose is more present then in other species, due to the high need of nervous system's cells metabolism [11]. The second most abundant carbohydrates are the: human milk oligosaccharides (HMO), complex molecules consisting in different combinations of monosaccharides, such as glucose, galactose, fucose, *N*-acetylglucosamine, and the sialic acid derivative N-acetyl- neuraminic acid [12]. HMOs reach the colon almost intact and act by giving several benefits to the child, working as prebiotic for the gut microbiota development, stimulating the growth of positive microorganism, like Bifidobacterium, having also an antimicrobical and antiviral

activities. The structure of HMOs is a decoy for viral particles, which bind to HMOs, instead of binding to cell surface's receptors and thus the infections are inhibited [9]. The type of HMOs present in milk is also subject to variations from mother to mother, and also depends on polymorphism in genes coding for enzymes, involved in the synthesis of oligosaccharides, for example fucosyltransferase 2 (FUT 2) [10] . Fifty percent of the nutritional requirements are covered by the lipidic component, therefore lipids have the most important role in providing nourishment, and they are also fundamental for the development of the nervous system and the retina, because of their structural role. Among lipids, 95-98% is made up of triglycerides, enclosed in lipid droplets. The lipases present in the child's saliva and digestive system transform triglycerides into monoglycerides and fatty acids. Some fatty acids, especially long-chain polyunsaturated fatty acids, such as docosahexaenoic acid (DHA), act on lipoxins, resolvins, protectins and maresins, with an intense immunomodulating and antimicrobial action. Derivatives from the oxidation of cholesterol have shown the capacity to inhibit some viruses, particularly widespread in pediatric population, like rhinovirus and rotavirus [9]. Lipidic concentration varies greatly based on diet, weight during pregnancy and breast fullness[13] .The protein fraction allows the absorption of amino acids essential for life and beyond: molecules of protein nature are circulating in the serum, such as alpha lactalbumin, lactoferrin, immunoglobulins, casein clots which exert many direct and indirect actions, related to immunitary protection at birth, and they are predominant in colostrum. More precisely, alpha-lactalbumin helps in lattose's synthesis, in the absorption of amino acids and trace elements, it binds to oleic acid forming human alfa-lactoalbumin made lethal to tumor cells (HAMLET), whose action is the object of studies [13]. Lactoferrin owes its name to its great affinity for iron, inhibits the growth of potentially pathogenic bacteria that require iron for their metabolism, has a bacteriostatic action, and works in sinergy with lysozyme. It has also antiviral activity, because it binds to the heparan sulfate domains present on the cell membrane and at the extracellular matrix level, hindering the binding of viral particles to the domains [9]. Immunoglobulins are components of maternal immunity, whose presence depends on a past or present infection; they are molecules that act towards specific organisms, and through breast milk, they also become available to the baby, who will most likely be in contact with the same environment. Among the

various immunoglobulins (A, E, D, M, G), secretory IgA are the most abundant in breast milk, covering the baby's gastrointestinal tract, exerting a local immune action, preventing the binding of specific microbial agents; in fact only the 10% of sIgA is transferred into circulation[13]. Breast milk is not sterile, unlike what was thought in the past. Cultured based studies and non-culture sequencing techniques have demonstrated the presence of a microbiota; a community of microorganisms transferred from mother to child and which contributes to the formation of the infant microbiota. The microbial ecosystem depends both on individual and environmental factors; in fact it has been difficult to identify microorganisms that are present in a stable way in different women's breast milk [13] . It is necessary to establish more systematic protocols to trace the composition of the maternal microbiota, because many discrepancies also depend on differences in sampling and storage techniques[13]. Three hypotheses have been formulated regarding the origin of the microbiota in breast milk: 1. It derives from the contact between mother's skin and baby's oral cavity and through the mechanisms of retrograde flow of milk from the baby to the breast 2. It derives from the colonization of the mammary gland by intestinal microbiota 3. It is transmitted by mother's skin. The most accredited hypothesis is the first, even if it has not yet been confirmed, because some bacteria found in children's feces and milk, are absent on maternal skin [13]. A study carried out by Collado et al. analyzed the microbiota of healthy mothers and infected mothers, noting that *Bifidobacteria* and *Lactobacilli* were present in uninfected mothers [13]. MicroRNAs are abundant in breast milk as free molecules or transported by vesicles. They remain intact in the intestinal tract and are absorbed by intestinal epithelial cells, reaching different organs through the bloodstream. They regulate gene expression in target cells, and therefore their differentiation and proliferation, contributing to developmental programming and immunoprotection [11]. Cytokines, growth factors, together with lactoferrin, lysozyme, Igs are molecules important for immunomodulation and passive protection, preventing disease occurrence. Growth factors are involved in the development of tissue and organs, such as intestinal tract, vasculature, nervous system, and endocrine system [11]. The hormones most present in breast milk are: insulin, leptin, ghrelin, apelin, nesfatin-1, obestatin, and adiponectin, and their role in infant growth is still under investigation.[11]

1.3 Defenses of the organism

In order to better understand the immunological factors present in the human milk, this paragraph is a synthetic description of the main immunological pathways that have evolved in humans to respond to infections[14].

Two main defensive mechanisms act to protect the organism from pathogenes:

1- innate immunity, that includes physical and mechanical barriers, microbioma, cells and chemical agents;

2- adaptive immunity, that is a specific response against specific microorganisms and their molecular components;

Innate and adaptive immune responses are connected and cooperate.[15]

1.3.1 Innate immune response

Skins, mucosal layers and the correlated enzymes and secreted acids are unspecific protective mechanisms, that hinder the persistence of pathogenes by the production of an unfavorable environment. Microbioma is also a protection, by limiting the growth of pathogenic bacteria through competition for nutrients and receptors.[16] Innate immunity cells (macrophages, dendritic cells, polymorphonuclear neutrophilis -PMN-, natural killer cells) and the chemical mediators (cationic proteins, complement system, cytokines such as IL1, IL2, IL3, TNF, INF alfa/beta/gamma) are able to trigger inflammatory responses and to eliminate pathogens. Innate immunity cells recognize molecular structures common to specific classes of pathogens (pAMPs- pathogen-associated molecular patterns) and recognize also intracellular molecules released by damaged cells (dAMPs- damage-associated molecular patterns) through specific cellular receptors such as Toll-like receptors (TLR) and soluble factors (PRR- pattern recognition receptors).[15] After the recognition phase, internalization and formation of the phagolysosome are the cellular and biochemical processes leading to the destruction of the pathogenes. For example, enveloped viruses can be attacked by defensins contained in the granules of PMN, and permealising the phospholipid membrane of the virus.[16] Innate immunity quickly triggers the inflammation

process, in case of infection, and activates the adaptive immunity response, that in turn enhances the innate response[15].

1.3.2 Adaptive immunity response

The defensive capacity and the breadth of the adaptive responses progressively increase with each subsequent exposure to a specific pathogen; in fact, the adaptive immune response is characterized by memory function. T and B lymphocytes, and the antibodies produced by B lymphocytes are the effectors of the adaptive response.[15] Adaptive immunity develops two or three days after the infection and reaches its maximum potential after few weeks: that is the frame of time necessary for the action of the innate response, for the antigens processing, for the presentation of antigens to T lymphocytes and for the antibodies' production[16].Antigens are molecular complexes of protein, polysaccharide and glycolipid capable of triggering a specific immune response. They are processed by some cells of the immune innate system, and by epithelial cells, and are presented on the cells' surface, through the major histocompatibility system I and II (MHC I, MHC II) in order to be recognized by lymphocytes T cells, both CD8+ and CD4+. Lymphocytes T CD8+ also known as cytotoxic lymphocytes, recognize the association of antigenic epitopes with MHC I and prevent the replication of intracellular parasites, such as viruses, by eliminating the host cell before the release of the pathogen. Lymphocytes T CD4+, called T helper, are stimulated by antigens presented by MHC II molecules and can activate two types of responses (fig.6)[16]:

1- cell-mediated immunity, in which cytotoxic cells (lymphocytes T CD8+, macrophages, memories cells of previous infection) massively attack infected cells, causing their lysis or apoptosis;

2- humoral immunity, in which B cells start the secretion of antibodies.

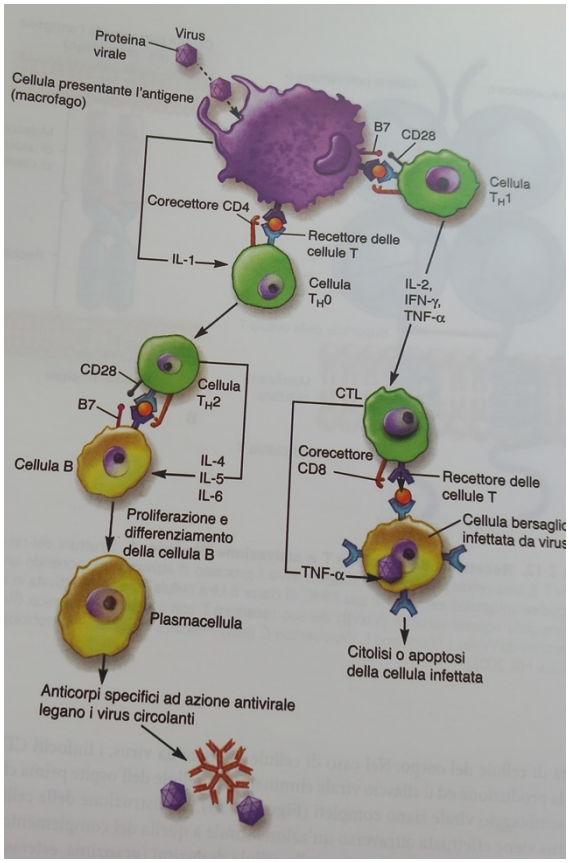


Figure 6. T cell responses. Medical Microbiology. J. Ryan Kenneth.

Antibodies belong to the family of immunoglobulins, that are proteins present in abundance in serum and on B cells' surface. Three different types of antibodies act against the infections: IgG, IgM and IgA. In general, the antibodies have a tetrameric structure, consisting of two light polypeptide chains and two heavy chains. The structure includes specific antigen recognition and binding sites; furthermore, antibodies are capable of activating effector functions through interaction with phagocytes, and other components, such as the complement system. Individuals without antibodies against a certain pathogen are defined seronegative or naïve, like naïve lymphocytes that have not yet encountered a specific antigen. Individuals with specific antibodies are defined seropositive. Antibody levels are variable but increase with each subsequent exposure.

The IgM are the first antibodies to be synthesized during the primary adaptive immune response, followed by the IgG. The IgA are the main component of local immunity,

aimed to protecting epithelial surfaces from colonization and infection, IgA molecules combine with other proteins (called secretory fragments -sIgA) and react with surface antigens of viruses and bacteria, preventing their establishment and local infections or invasions of subepithelial tissues. Figure 7 represents the main events of antibodies production, that are the primary response to a new antigen and the developing of the secondary response after a subsequently exposure to the same antigen.[16]

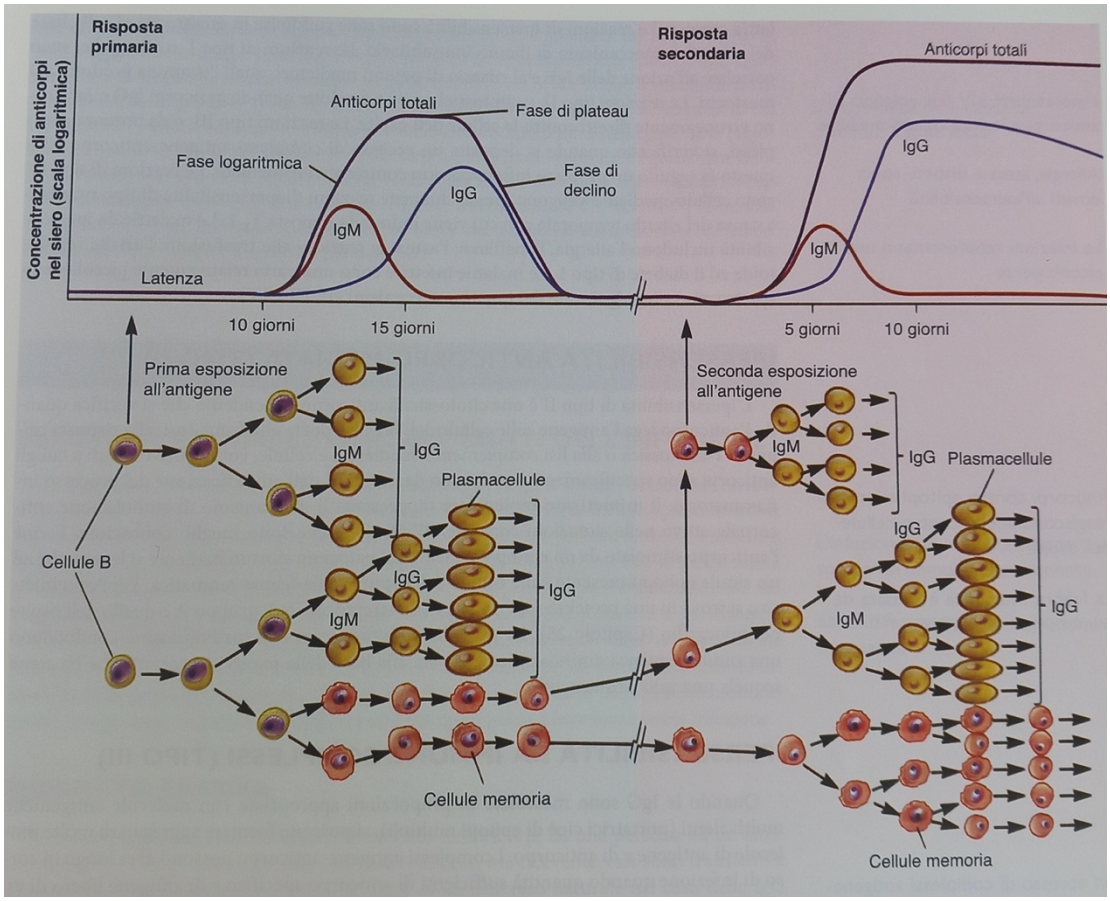


Figure 7. Kinetics of antibody production. Medical microbiology. J. Ryan Kenneth

1.3.3 Innate immunity and breast milk.

At birth, the infants' immune system is immature, because the physical and chemical barriers are not yet fully developed, innate immunity cells are poorly active, production of AMPs is limited, the complement system is not complete, anti-inflammatory mechanisms (in particular of the intestinal and respiratory tract) are insufficient. Based on this, all the defensive components of human milk are crucial [17] . Innate immune cells contained in human milk are: macrophages, neutrophilis, lymphocytes,

hematopoietic progenitors cells and hematopoietic stem cells. Soluble TLR (sTLRs), soluble CD14 (sCD14s), human-defensin, act as PRR.

Macrophages that are particularly high concentrated in colostrum and in general at early lactation, they act as phagocytes, without initiating a significant inflammatory response: a balance between the inflammatory response and the inhibition of the inflammatory response is very important for child's health and development, because it balances protective effects of inflammation with protection of tissues from being damaged. Soluble TLR and CD14 cells interact with other compounds in breast milk, regulating the inflammatory response. Several human milk's components, such as caseins and lactoferrin, have an antimicrobial activity and anti-inflammatory effects [17]. Additionaly, milk microbiome and its nutrients (such as HMOs) represent defensive systems, because they are able to compete with potential pathogens and to contribute to the formation of intestinal mucus layer, that in turn is important in reducing the antigenic contact between epithelial cells and potentially pathogenic bacteria[17].

1.3.4 Adaptive immunity and breast milk

During the first months of life the molecular mechanisms of adaptive immunity are formed, leading to the distinction between self and non-self antigens[16], while the response against foreign antigens is impaired. T cells of newborns promote self-tolerance and the immune response has mainly an anti-inflammatory profile. B cells have impaired function, so the secretion of immunoglobulin is delayed and limited, but breast milk is one of the vertical transmission mechanisms that allow the baby to receive maternal antibodies [9]**.** Transitional milk shows higher concentrations of IgA and IgM, while IgG are predominant in mature milk [17]. Plasma cells associated to the mammary glands produce IgA that are contained in breast milk and cellular transports mechanisms of mammary epithelium allow the release of immunoglobulins into breast milk.

1.4 Antiviral activity of human milk compounds

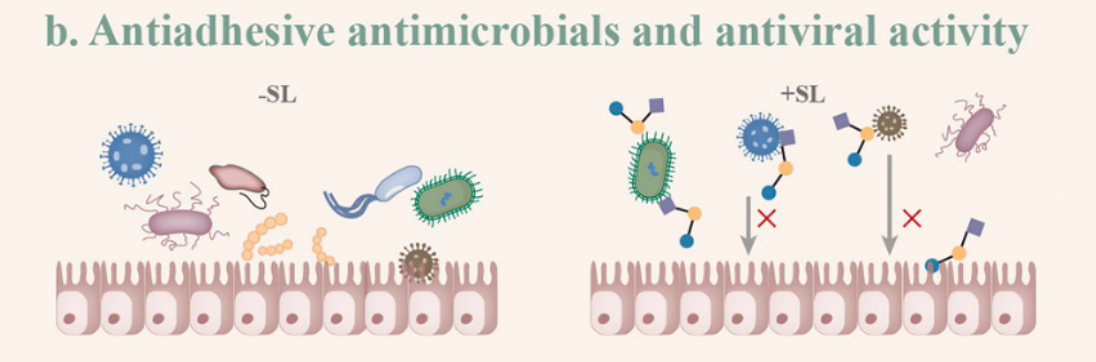


Figure 8. Physiological effect of sialylated oligosaccharide: antiadhesive antimicrobials and antiviral activity. Yingying Zhu et al.

Human milk contains several molecules with antiviral activities, some of them have been confirmed through *in vitro* and *in vivo* models, while other only *in vitro* and need further investigations. Human Milk Oligosaccharides (HMOs), glycosaminoglycan, lactoferrin, lactadherine, mucins, tenascin-C, lipid compounds, extracellular vesicles, beta-lactoglobulin, alfa-lactalbumin, and immunoglobulins have shown antiviral activities thorough different molecular mechanisms[18]. Our present study is focused on oligosaccharides, lipid compounds and immunoglobulin contained in human milk and further investigating the already known antiviral activities. The molecular structure of HMOs gives them the capacity of mimicking the viral receptors on host cells, thus leading to the inhibition of viruses-host attachment, and to the reduction of the infection rates (fig.8). For istance, Noroviruses, are able to bind some histo-blood group antigens (HBGA), expressed on epithelial cells of the gastrointestinal tract, and also to the 2′-fucosyllactose (2'FL) human milk oligosaccharide, that is a biosynthesized oligosaccharide, structurally identical to those contained in breastmilk, at least *in vitro*. Two strains of norovirus are subjected to a reduction of their capacity to bind their host receptors, in presence of 2'FL[19]. Other sialylated and fucosylated human milk oligosaccharides, such as 3'-sialyllactose (3'SL), 6'-sialyllactose (6'SL), 2'-fucosyllactose (2'FL), have shown the ability to reduce the infectivity of human rotavirus *in vitro*: 2'FL added after the onset of the infection produces a reduction of

infectivity of 62%, while the mixture of 3'SL and 6'SL, added during the infection, gives the 73% of infectivity reduction[20] (fig.9).

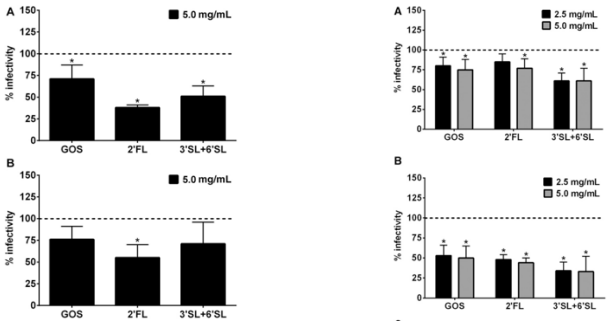


Figure 9.A-B (left side): addition of oligosaccharides after virus absorption reduces the infectivity of human rotavirus strains in MA104 cells. A-B (right side): preincubation of virus for 2 h in the presence of milk oligosaccharides (2#FL, GOS, 3#SL, and 6#SL) reduces the infectivity of human rotavirus strains G1P[8] (A) and G2P[4]. Laucirica D.R. et al.

Respiratory viruses, such as the respiratory syncytial virus (RSV), influenza A virus (IAV-H1N1), severe acute respiratory syndrome virus-2 (SARS-CoV-2), have exhibited less capacity to infect permissive respiratory cell lines that have been treated with both sialylated and fucosylated human milk oligosaccharides before the inoculum (pre-treatment)[21] (fig. 10).

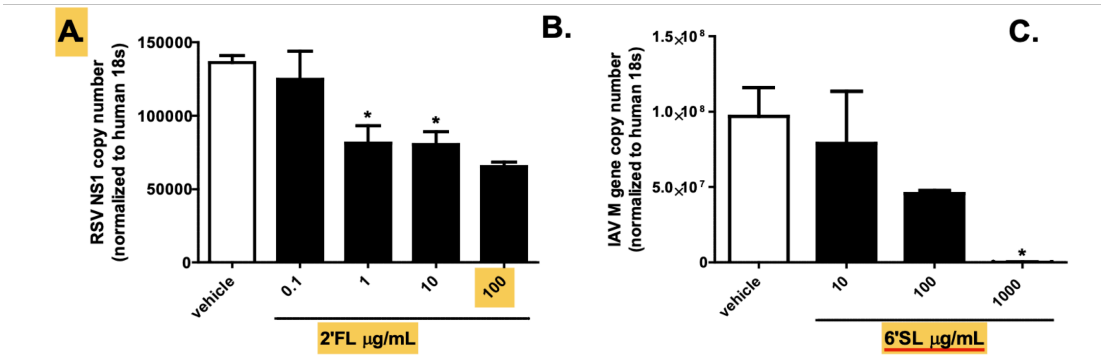


Figure 10. HMO effects on Calu-3 epithelial cells. Duska-McEwen G. et al.

Fatty acids isolated from human breast milk have also exhibit antimicrobial properties, particularly against lipid-coated microorganisms, such as enveloped viruses: hepatitis C virus (HCV), influenza virus, herpes simplex virus (HSV), respiratory syncytial virus (RSV)[22]. The hypothesized molecular mechanism of inhibition consists in the disruption of the viral envelope, caused by the incorporation of free fatty acids, diglycerides and monoglycerides, released from the milk fat globular membrane (MFGM) (fig.11).

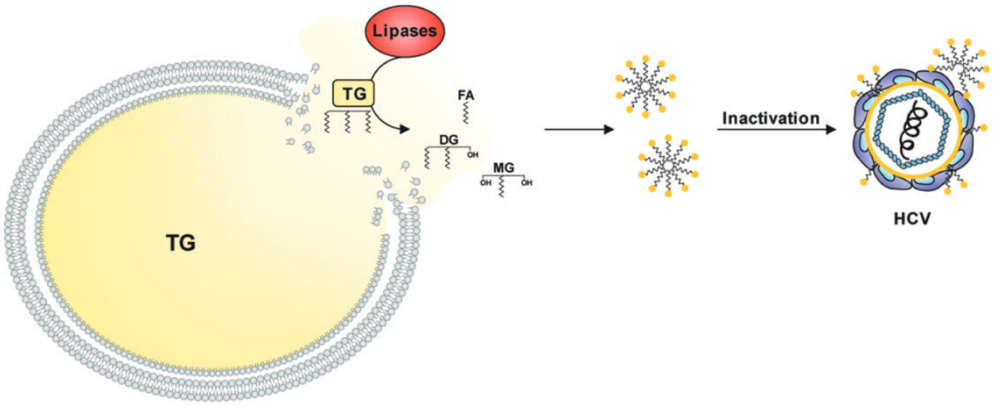


Figure 11. Hypothetical model for inactivation of HCV by human breast milk. Pfaender S. et al. (TG: triglyceride core).

Among the milk's compounds tested on Zika virus' s infections, fatty acids have shown to reduce the viral titers, in i*n vivo* studies[23].

Several studies concerning SARS-CoV-2 antibodies in human milk showed the presence of neutralizing IgA, IgM and IgG in breast milk of positive, vaccinated mothers and of mothers who have previously been infected[24]. However, also breast milk from negative, unvaccinated mothers exhibits neutralizing activity, probably due to the presence of aspecific compounds with antiviral effect[25]. The presence of IgA anti-SARS-CoV-2 seems to be fundamental for the neutralization; one study focused on the specific contribution of each class of Immunoglobulins and found out that anti-SARS-Cov-2- IgA's depletion completely abolished the ability of human milk to neutralize SARS-CoV-2[26] (fig 12).

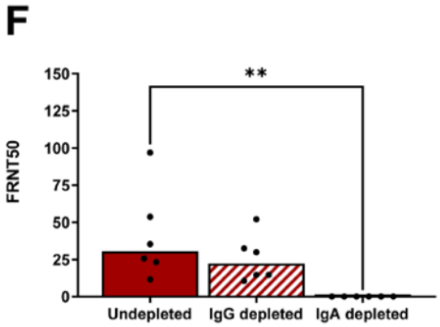


Figure 12. FRNT50 values after IgG or IgA depletion. Macchiaverni P. et al.

The antiviral defensive action of antibodies can occur through different molecular mechanisms: inhibiting the bond between virus and host cell, inhibiting the fusion between virus and cell membrane, blocking the viral genome uncoating step and inhibiting the transcription of viral genes[27].

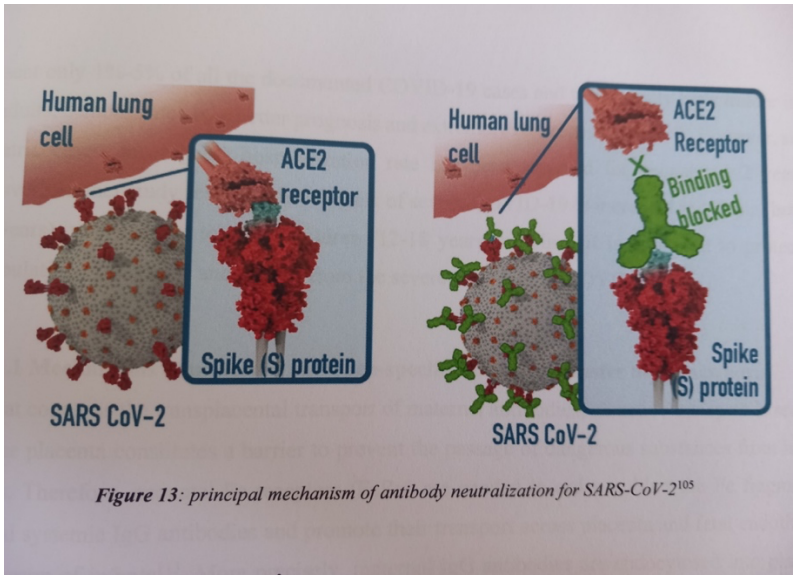


Figure 13. principal mechanism of antibody neutralization for SARS-CoV-2.

Immunoglobulins of different isotypes are selectively distributed in the different body areas; dimeric IgA are predominant in the secretions of the mucosal epithelia, in fact breast milk is rich in secretory IgA (sIgA), with a concentration higher than in serum (fig.14)[28]. Through breastfeeding the baby is protected by the IgA contained in milk, as a study conducted on norovirus demonstrated: high amount of norovirus-IgAs deriving from the breast milk protected the infants from severe symptoms (i.e. severe diarrhea)[29]

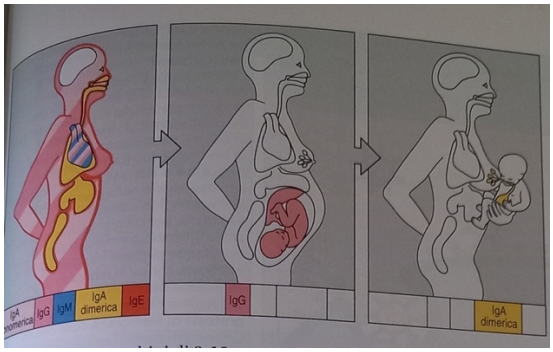
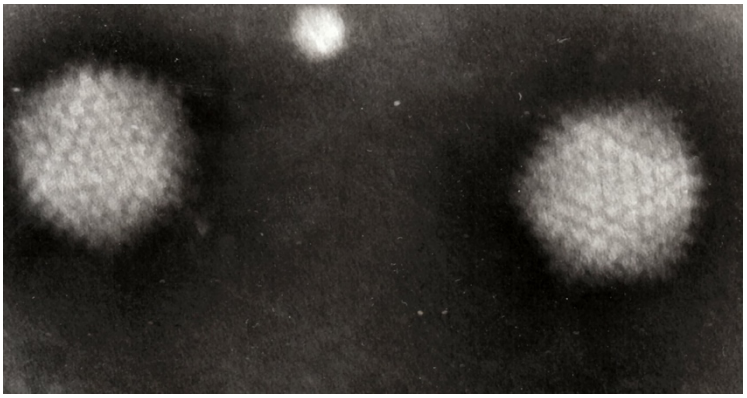


Figure 14. Immunoglobulin's distribution. Parham P. "The Immunity system".

1.5 Human adenovirus

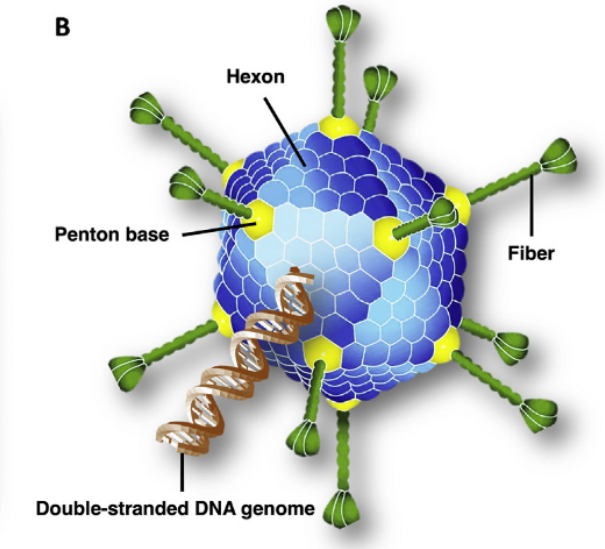


Human adenoviruses (HAdVs) belong to the *Adenoviridae* family; seven different species have been classified (A-G) depending on genetic, physical and chemical properties, such as the percentage of guanine plus cytosine in the DNA, the DNA homology and the length of the fibers[30] . The initial isolation from human adenoid in 1953 gave the name to the taxa. Adenoviruses can infect cells of the respiratory, gastrointestinal, ocular and urinary tracts; most of the infections are subclinical, causing symptoms similar to common cold, but the spectrum of diseases is wide and the pediatric population is the most affected [31].

1.5.1 Genome and structure

HAdVs are double-stranded DNA viruses, without envelope, with icosaedric symmetry, 70-100 nm in size [30]. The genome is linear and variable in length, between 26-45 kbp among different species, and is condensed in the virion's core; the viral polypeptide VII is important for the stability of the core[30]. The capsid consists in 252 capsomers: 240 hexon trimers, 12 pentameric pentons from which 12 fibers extend. The antigenicity of hexons is common among all HAdVs, while pentons carry species-

specific antigens and fibers contain type-specific antigens and are responsible for hem-



agglutination activity[30].

Figure 15. Structure of adenovirus virion. Wun-Ju Shieh.

The genome organization consists in inverted terminal repeat sequences (ITR) of 100 bp at each end of the linear genome, in which two replication origins are located (fig.9). A virus-coded protein is covalently bonded to both 5'-end (55-kda terminal protein, TP). The replication of the viral genome requires the products of E2 genes (leftward-oriented promoters): the precursor of TP (ptp), adv DNA polymerase (adv Pol), and the DNA-binding protein (DBP). Two cellular transcription factors (NFI and Oct-1) participate in the replication initiation, enhancing it. At the end of replication, the ptp is cleaved by a viral protease to TP. The HAdVs genome organization is higly conservated among different species and consists in five early transcription units (E1A, E1B, E2, E3, E4), intermediate transcription units (IX, IVa2, L4 intermediate, E2 late) and a major late transcription unit (MLTU, L1-5, rightward-oriented promoters) genes: different regions which encode for proteins necessary respectively at early, intermediate and late stages of the viral replication. E3 is the most divergent gene between the different HAdVs and probably one of the cause of pathogenicity and tropism variations[3233]

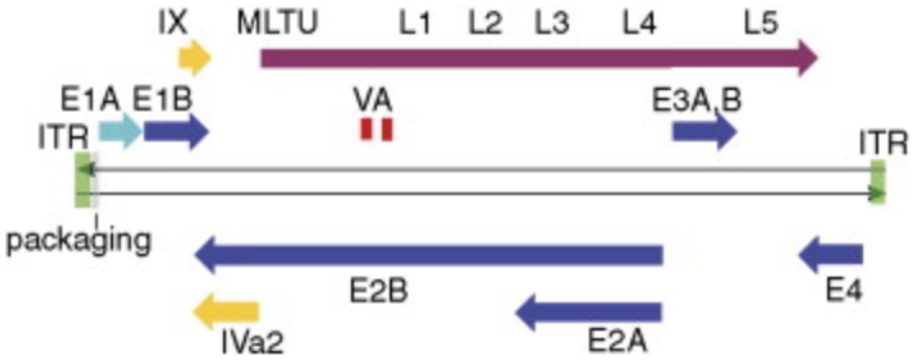


Figure 16. Gene and genome features of human adenoviruses. Adenoviruses. K.N. Leppard

1.5.2 HAdVs replication cycle

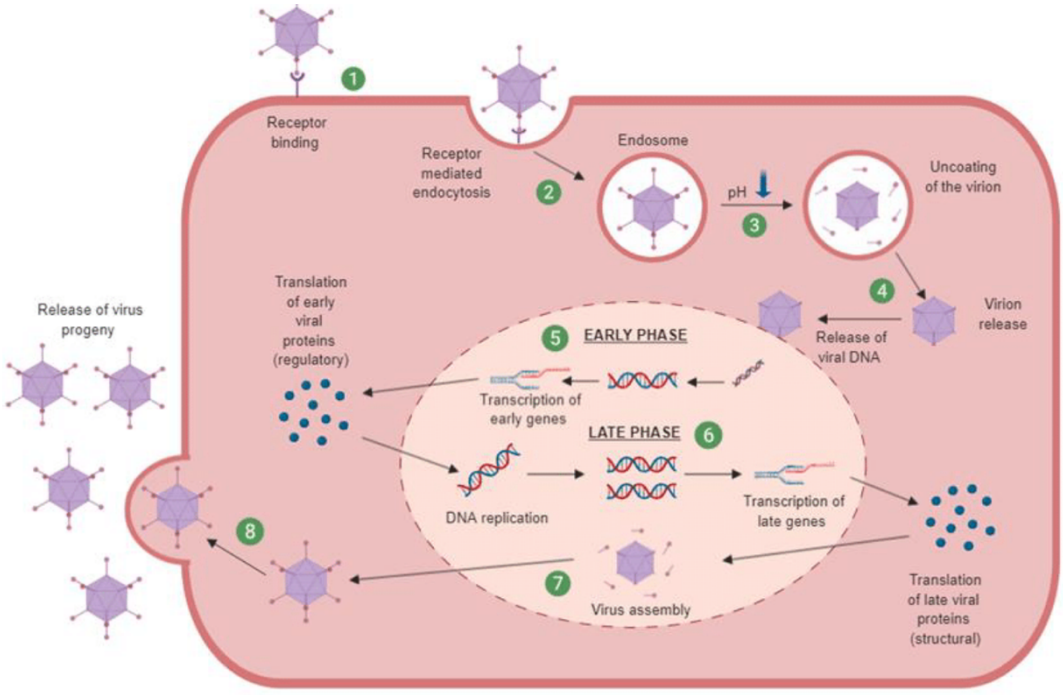


Figure 17 Adenovirus replication cycle. Alan L Parker

Viral fibers extending from the capsid are the structures that allow the virus to attach to host cells[27]. Many HAdVs species (A, C, D, E, F) have high affinity for the host-cell transmembrane CAR protein (coxsackie B-adenovirus receptor), which is abundantly expressed in a variety of tissues. B and D species bind CD46, a regulator of the complement cascade present on the plasma membrane of most cell types, including hematopoietic cells.

After the attack of the virus on the host cell membrane, the internalization phase follows, mediated by interactions between the base of the pentons and the cellular

integrins and then the virus enters by endocytosis. The high acidity of the endosome leads to the rapid release of the partially degraded capsid, then microtubules are probably involved in the transport of the viral particle, through the cytoplasm, to the nucleus which is the site of virus transcription, DNA replication and assembly.

The early E1A gene product acts by modulating the cell cycle; infact, early events have the effect to induce the host cell to enter the S phase of its cell cycle, and in turn to create favorable conditions for viral replication[27]**.** The early gene E1A also activates the transcription of the other early genes. The early E1B region encodes proteins that block cell death (apoptosis), avoiding premature death of the cell[27]. With the onset of DNA replication, intermediate genes are transcribed and the late events begin simultaneously, i.e. the expression of the late genes (L) which code for the viral structural proteins. A single large primary transcript (29,000 nucleotides in length) is then processed, and alternative splicing, or alternative polyA, generate at least 18 different late messenger RNAs (mRNAs); mRNAs are divided into groups from L1 to L5. The transcripts are transported to the cytoplasm where viral proteins are synthesized. A complex consisting of the E1B polypeptide and the E4 polypeptide inhibits the cytoplasmic accumulation of cellular mRNAs and promotes the accumulation of viral mRNAs[27]. VA genes express noncoding RNAs involved in the facilitation of viral late mRNAs translation and also in reducing the cellular interferon antiviral response[30].

Virion morphogenesis occurs in the nucleus. Each hexon capsomer is a trimer made up of identical polypeptides; the penton is composed of five core polypeptides and three fiber polypeptides, a late scaffold protein, encoded by L4 participates in the aggregation of hexon polypeptides, but is not part of the final structure. The capsomers aggregate spontaneously to form empty capsid shells and subsequently the naked DNA enters the capsid through a DNA element that serves as a signal for packaging and the action of scaffold proteins[30]. The assembly process is inefficient, approximately 80% of the hexon capsomeres and 90% of the viral DNA are not used and accumulate in the nucleus and cause the characteristic nuclear lesions of HAdVs infections. Infected cells lyse and release viral particles[31]. The infectious cycle of the adenovirus lasts approximately 24 hours and approximately 100,000 viral particles are produced by each cell[27].

1.5.3 Pathogenesis

The infection can occur in the epithelial cells of the respiratory tract, conjunctiva and cornea, gastrointestinal tract, urinary tract and can establish persistent and latent infections in tonsillar and intestinal lymphocytes (in particular HAdV species C)[30]. The cell lysis caused by the infection generates damage to the epithelial mucosa and parenchymatous tissue, inducing the activation of the host's inflammatory processes; pattern of necrotizing inflammation can develop.[31]

HAdVs infections are mostly mild and heal spontaneously[30]. Three per cent to 6 per cent of common cold in children are caused by HAdVs, but HAdVs can cause also more severe respiratory infections: 5% of the acute respiratory diseases, such as pharyngitis, in children, are caused by HAdVs, more frequently by species C, type 1, 2, 5. Adenoviral pharyngitis is characterized by fever, sore throat, extensive exudative tonsillitis, and frequently, cervical adenopathy, headache, myalgia, chills, malaise, cough, nasal congestion and abdominal pain[31] and, particularly in closed environment, can become an epidemic illness. HAdVs types 3, 7, 21 are thought to be responsible for approximately 10-20% of pneumonia cases in childhood; more precisely, severe pneumonia affect more commonly neonates and young children, from 3 to 18 months of life [31]. Pericarditis and myocarditis associated with severe adenoviral pneumonia, in children, have been reported[31]. In 2007, there was an epidemic of severe respiratory disease, caused by a new variant of adenovirus 14, affecting patients of all ages, including healthy young adults. Respiratory-pharyngeal syndromes caused by adenovirus can also affect the ocular region, with consequent symptoms that include fever, conjunctivitis and pharyngitis: pharyngo-conjunctival fever is associated with types 3 and 7 and tends to occur in epidemic form ("swimming pool conjunctivitis"). Seventy-five per cent of cases of common conjunctivitis (“red eyes”) are caused by adenovirus infections, generally subclinical and moderately symptomatic[30].

A serious disease is the keratoconjunctivitis, caused by types 8,19, 37, which most frequently infect the adults. It consists of acute conjunctivitis, followed by keratitis[30,31]. Serotypes 40 and 41 have been etiologically associated with childhood gastroenteritis and are responsible for 5-15% of cases of viral gastroenteritis in young children[30].

Immunocompromised patients, transplant patients, patients suffering from acquired immunodeficiency syndrome, may contract systemic infections of variable severity[30].

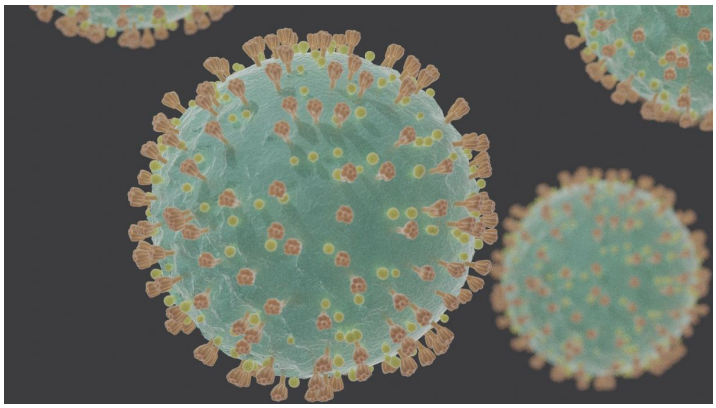
Acute hemorrhagic cystitis can be caused in children and more common in males, by types 11, and 21; the incidence is low in immunocompetent individuals and is the only syndrome that appears to be related with sex[31].

1.5.4 Epidemiology

Adenovirus is ubiquitous in human and animal populations, survives long periods outside a host, is worldwide distributed and infections are more common in winter or early spring, but can also occur in other seasons[34,35]. The infections and associated diseases can be sporadic or epidemic. The most common serotypes in clinical samples are the respiratory and gastrointestinal types[30]. Adenoviruses transmission can occur through aerosolized droplets, fecal-oral route, direct inoculation to the conjunctiva, exposure to infected tissue or blood of infected person, can also occur from exogenous sources (pillows, lockers, towels, ophthalmic solutions, swimming pool water) and reactivation[35]. The incidence of HAdV infection is higher in infants and children between 6 months and 5 years of age. Infections with types 1, 2, 5 are mainly found in the first years of life, while types 3, 7 at school age[31]. Cases of epidemic forms of acute respiratory syndrome, caused by types 3,4,7, are frequent among military recruits, in conditions of high stress and crowding. Military barracks, public swimming pools, household with young children, medical facilities, daycare centers are closed and often crowded places where the incident of infections is more frequent.

Adenovirus vaccine is available only for U.S. military[34]. Good hygiene practices are very important to prevent infection and the spread of the virus[34,35].

1.6 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and its epidemiology



Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is part of the family C*oronaviridae*, subfamily *Orthocoronavirinae* and belongs to the *Betacoronavirus* genus. *Betacoronavirus,* together with *Alphacoronavirus* genus, includes coronaviruses that are a source of infection mainly for mammals[36]. SARS-CoV-2 is the seventh CoV identified able to infect humans, and along with severe acute respiratory syndrome (SARS-CoV), is part of the species SARS-related coronavirus, subgenus *Sarbecovirus*, whereas Middle East respiratory syndrome coronavirus (MERS-CoV) belongs to the subgenus *Merbecovirus*[37]. In 2002 SARS-CoV disseminated to 33 countries, causing SARS disease: 8000 cases in 33 countries in eight months, with a global fatality rate of 10%[38]. MERS-CoV was firstly identified in Saudi Arabia, in 2012, disseminated to 27 countries and WHO reported approximately 35% of fatal cases[39]. SARS-CoV-2 was firstly identified in Wuhan, Hubei province of China, at the end of 2019, after a sudden spread of pneumonia of unknown cause, epidemiologically linked to a local seafood market in which alive wild animals were available for sale[40]. The disease caused by SARS-CoV-2's infection has been named coronavirus disease 19 (COVID-19) by WHO, and on March 11 th, 2020, has resulted in a global pandemic[41]. The majority of human coronaviruses have zoonotic origin, and more precisely, bats seem to represent the animal reservoirs. Bats are considered animal reservoirs because bat SARSr-CoV (RaTG13) shows high sequence similarity to SARS-CoV-2 (96.2% genome similarity) and also other bat -SARS-related coronaviruses (RmYN02, RpYN06 and PrC31) are similar to SARS-CoV-2 in specific genomic regions, such as ORF1ab[42]. Two sub-lineages of SARSr-CoVs isolated from

Malayan pangolins, show a modest sequence similarity across the whole viral genome but a high nucleotide identity in regions that are essential for the interaction with human cellular host, such as the region encoding for the receptor binding domain of the spike protein (RBD)[43,44]. In summary, bat SARSr-CoV could be the evolutionary source of SARS-CoV-2 or alternatively they probably share a common ancestor; another possibility is that SARS-CoV-2 evolved from SARS-CoV in humans, after spreading from an animal source[42]. Since 2020, several variants of SARS-CoV-2 have emerged, due to random mutations in the viral genome, that alter the virus's pathogenic potential. The WHO identifies and tracks SARS-CoV-2 variants of concern (VOCs), SARS-CoV-2 variants of interest (VOI), SARS-CoV-2 variants under monitoring (VUM). Five of the main SARS-CoV-2 VOCs were identified since the beginning of the pandemic: [45]

1. Alpha (B.1.1.7) first variant of concern described in the United Kingdom (UK) in late December 2020;
2. Beta (B.1.351): first reported in South Africa in December 2020;
3. Gamma (P.1): first reported in Brazil in early January 2021;
4. Delta (B.1.617.2): first reported in India in December 2020;
5. Omicron (B.1.1.529): first reported in South Africa in November 2021

All the five listed VOCs have mutations in the RBD and NTD coding regions, thus leading to increased affinity of the viral spike protein to the Angiotensin converting enzyme (ACE2) receptor of host cells.

Considering the epidemiological update of the European Centre for Disease Prevention and Control as of 30th of August 2024, there are no major SARS-CoV-2 variants meeting the VOC criteria[46].

SARS-CoV-2 way of transmission among humans includes aerosol, respiratory droplets, surface contamination and also the fecal-oral route has been considered a potential route of human transmission. Nonsymptomatic/presymptomatic individuals are able to transmit the virus. Mask-wearing, contact tracing, physical isolation are the disease control measures, applied to contrast viruses spread[47]. M-RNA vaccines, viral vector vaccines, protein subunit and inactivated virus vaccines have been developed and available since 2021[48].

Coronaviruses cause multiple respiratory and intestinal diseases of various magnitude; SARS-CoV, MERS-CoV, SARS-CoV-2 primarily cause respiratory infections[44], that can lead to viral pneumonia in some patients. All age groups are susceptible to the infection, but pediatric population have a more favorable clinical course than other age groups[49]. Concerning breastfeeding and SARS-CoV-2 infection, the CDC invites mothers with infection or suspected COVID-19, to breastfeed their babies, if in good clinical condition, but applying all the correct hygiene rules (precautions from contact and droplets)[50].

1.6.1 Genome and structure

Coronaviruses are enveloped, spherical, positive-sense single stranded RNA viruses. The name is due to the external structural proteins which produce the crown-like morphology[51] (fig.18). The diameter of the viral particles is about 120 nm. The linear RNA genome ranges from 25 to 32 kilobases in length, is associated with the N proteins to form the nucleocapsid, it functions as both a genome and an mRNA and includes ten open reading frames (ORF). Approximately two thirds of SARS-CoV-2 genome are occupied by the two largest ORFs, ORF1a and ORF1b, at the 5'terminal, which encodes for two co-terminal polyproteins pp1a and pp1ab; pp1a and pp1ab produce, by autoproteolytic process, 16 non-structural proteins, such as the RNA-dependent RNA polymerase (RdRP)[52]. One third of the genome, at the 3'terminal, include ORFs that encode for the structural proteins: spike glycoprotein (S), envelope protein (E), membrane glycoprotein (M) and nucleocapsid (N). Eight additional small ORFs, inserted between the structural genes, encode for accessory proteins which have a regulatory activity in the viral infection but are not included into the virion particle[53]. The surface structural proteins (S, M, E) are incorporated in the lipid bilayer that derives from the host membrane. The S proteins extends from the envelope, giving the crown-like shape to the viral particles, and is composed by two subunits: subunit 1 (S1) contains the receptor-binding domain (RBD) that binds to ACE2 on the surface of host cells, while subunit 2 (S2) mediates the fusion between the membrane of the virus and the host cell. More precisely, the functional domains of S1 subunit are the RBD domain, the amino-terminal domain NTD and two carboxy-terminal domains (CTD1 and CTD2), whereas the S2 subunit contains a fusion peptide, two heptad

repeats (HR1 and HR2) and a transmembrane region[53]. Comparing with SARS-CoV, the S protein of SARS-CoV-2 has five of the six residues crucial for binding to ACE2, that are mutated, and at the boundary between S1 and S2 subunits, a new insertion of four amino acids residues is present in SARS-CoV-2[54].

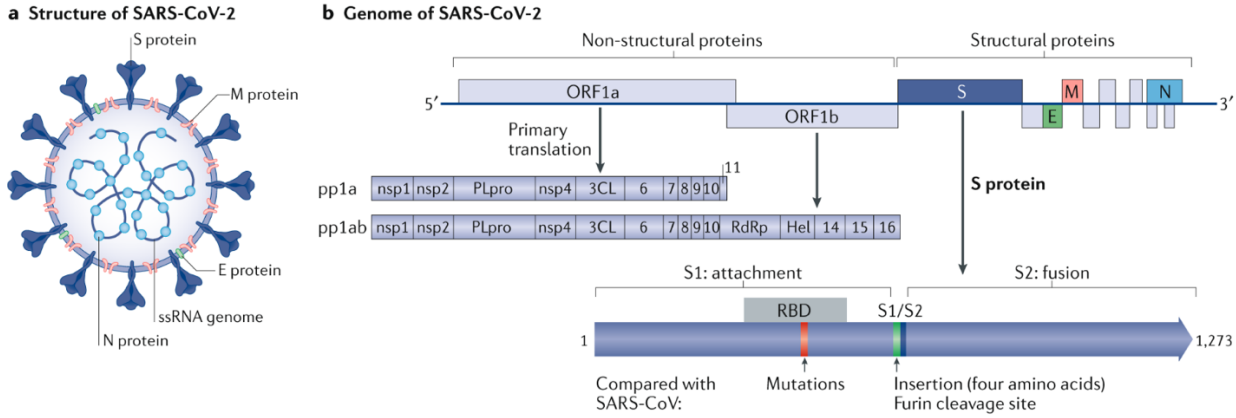


Figure 18. Structure, genome of SARS-CoV-2. Masataka Nishiga et al.

1.6.2 SARS-CoV-2 replication cycle

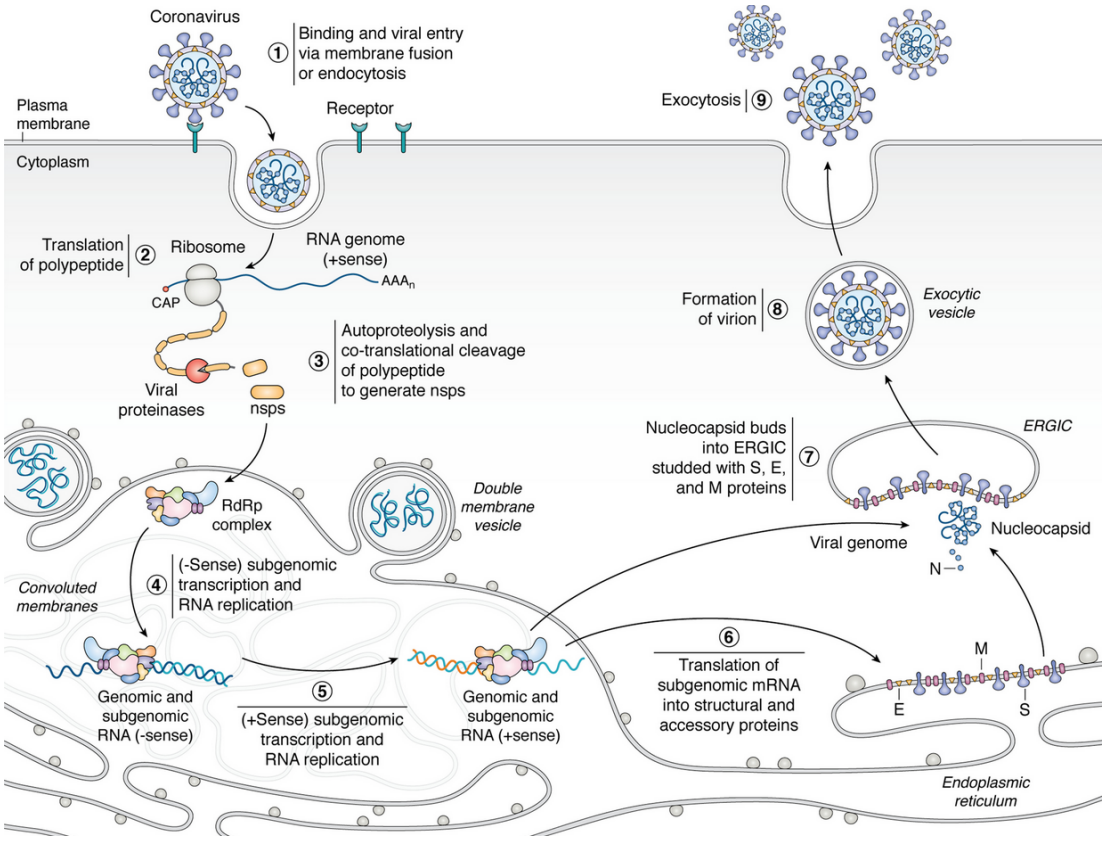


Figure 19. SARS-CoV-2 replication cycle. Ella Hartenian et al.

The viral replication cycle occurs mainly in the cellular host's cytoplasm. The first step of the infection consists in the attachment of the virus to the host cell membrane through specific interactions between S protein and the host receptors, thus leading to the endocytosis of the virus into the host cell or direct membrane fusion. Host proteases (transmembrane serine protease 2 TMPRSS2) cleaved the S protein, leading to the activation of the S2 domain and to the fusion between the viral particle and the cellular host membranes[55]. Subsequently, the viral RNA genome is released into the host cell cytosol and ORF1a and ORF1b are immediately translated by host ribosomes into polyproteins pp1a and pp1ab; the subsequent proteolytic cleavage of pp1a and pp1ab leads to the production of the individual non-structural proteins (nsps), which then form together the replication-transcription complex (RTC), necessary for the viral replication[51]. RTC copies and transcribes the viral genome, within protected microenvironment formed by double-membrane structures, that consist of virus-induced double-membrane vesicles (DMVs), small open double-membrane spherules (DMSs) and convoluted membranes (CMs) derived from the endoplasmic reticulum (ER)[55]. In this context, through RTC's activity, both genome-length RNAs and negative-sense subgenome are generated, that are then used as template for the synthesis of positive-sense full-length progeny genomes and subgenomic mRNAs. The synthesis of the negative-sense templates for the positive-sense sub-genomic mRNAs occurs through a process that has been named "discontinuous transcription"(fig.20); in this model the viral polymerase (RNA-dependent RNA polymerase) starts the negative-strand synthesis at 3'end of the positive-sense genomic template, pauses at internal transcription regulating sequences (TRS), dissociates from the genome strand and binds to the homologous TRS element downstream[55]. Then, all the positive-sense mRNAs are translated into structural and accessory proteins[55].

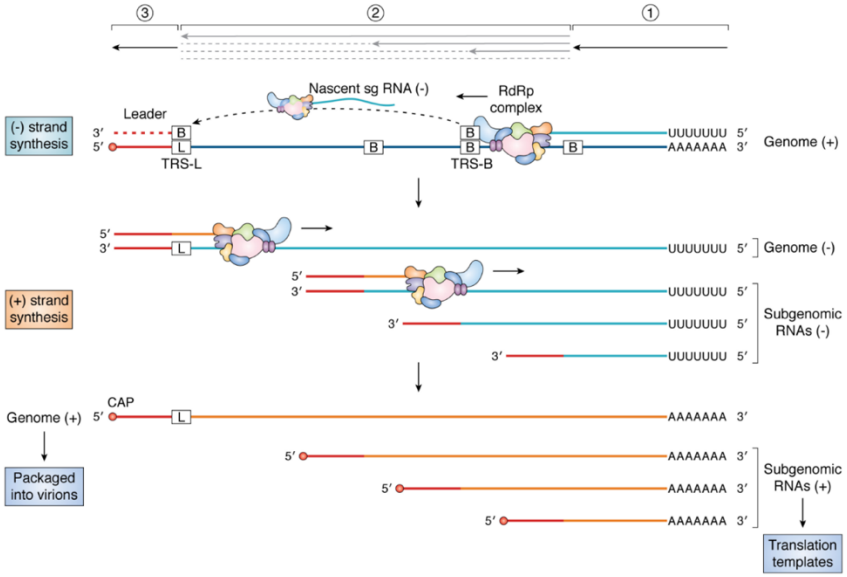


Figure 20. Discontinuons transcription. Ella Hartenian et al.

The viral structural proteins incorporated into the envelope (S, E, M) are translated in the endoplasmic reticulum (ER) and kept into its membranes at the site of budding; after the budding from ER, the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) transits through the secretory pathway and the nucleocapsid core of the virion newly produced, moves from the RTC and buds into the ERGIC membranes, which carries protein S, E, M, thus leading to the formation of mature virion. After the assembly, the newly enveloped virions leave the cell through the exocytic pathway[55] (fig.19).

1.6.3 Pathogenesis

Coronavirus disease 2019 (COVID 19) is the respiratory disease cause by SARS-CoV-2. SARS-CoV-2 infects epithelial cells in the upper respiratory tract, such as multi-ciliated epithelial cells in the nasopharynx and trachea and columnar epithelial cells of the olfactory mucosa. The infection can spread to the lower respiratory tract, to lungs alveolar type 2 cells and also to other organs[56]. Studies regarding the expression of ACE2 and of TMPRSS2 show how the cellular tropism of SARS-CoV-2 can be wide: gastrointestinal tract, kidney, heart muscle and central nervous system[57,58]. The clinical manifestations can also be different, ranging from mild cases with symptoms similar to the common cold, to more severe cases of interstitial pneumonia and acute respiratory distress syndrome (ARDS) that is the principal cause of death in patients with COVID-19. The rapid infection of the alveolar type 2 cells in lungs can cause a

strong inflammation response and injury due to high production of pro-inflammatory cytokines (cytokine storm). In this contest, the vascular permeability increases and there is a reduction of gas exchange, causing hypoxaemia. When the gas exchange is completely compromised, the use of invasive mechanical ventilation and/or extracorporeal membrane oxygenation becomes mandatory. Prothrombotic complications due to SARS-CoV-2 infection have been detected and are under study and investigation[57].

The most common symptoms are fever, cough, myalgia, tiredness while serious symptoms include difficult breathing or shortness of breath, chest pain or pressure, loss of the capacity to speech or move. People of older age (>60) and with co-existing and pre-existing diseases have higher risk of developing ARDS, so age is a major risk factor. Considering a disease spectrum in a group of 44,672 cases of COVID-19, diagnosed in Hubei province, the following percentage were detected: 81% of mild disease, 14% of severe, 5% of critical and deceased[56].

1.7 Evolutionary aspects and hypotheses about lactation and human milk

The specific feature of Mammalia is the production of secretions by the female individuals, which nourish and protect the offspring after birth, and during the first months of life[59]. *Homo sapiens* belongs to the Primate order, Mammalia class and to placentals mammals (Eutheria)[60]. Regarding the evolution of human milk and the evolution of mammals in relation with lactation, interesting hypothesis have been developed. Several evidences support the hypothesis that the immunological function of milk evolved before, then the nutritional one, and that the mammary gland evolved from a protective skin gland able to produce mucous secretion rich in antimicrobial molecules[61]. Many protective molecules such as lactoferrin, transferrin, lactoperoxidase, defensins, complement proteins are essential effectors of the innate immune system and are also found in the mammary gland and milk: since the innate immune system has evolved before the advent of mammals, it is reasonable to consider that milk has evolved as an antimicrobial secretion. Moreover, xanthine dehydrogenase and lysozyme, both involved in protective and signaling processes, have shown also essential functions in the development of the mammary gland: experiments of targeted disruption of Xanthine OxidoReductase (XOR) in mice show

that in heterozygous mice (XOR +/- females) the mammary epithelium collapses, with a premature involution of the mammary gland and incapacity to maintain lactation[62]. Knock-in mutations in kinases involved in the activation of NF-κB have highlighted the role of Nf-κB in the differentiation of the mammary epithelium during pregnancy and in the correct development of breastfeeding[61]. Nf-kb is one of the elements involved in the regulation of XOR expression. XOR is also essential in the milk fat droplets enveloping and secretions, and therefore is an example of "gene sharing" and of the evolution of additional functions[61]. Focusing on the evolution of mammals, breastfeeding probably had been advantageous at the end of the Mesozoic, when climatic and floristic changes had probably made harder the foraging, especially for young individuals. In that context, a type of parental feeding in which parents provide the nourishment to the offspring, by suckling or parental processing of food, was beneficial. Furthermore, breastfeeding, as other forms of parental processing of food, enables the young individuals to have a diet similar to that of adults, in a short time, while for reptiles of similar size, different diets are needed, based on the different growth stages[59]. Lactation seems to have made possible the development of diphyodonty, delaying the eruption of teeth and giving time for a sufficient development of the jaw[59].

AIM OF THE STUDY

The partially immature immune system of infants requires support that is acquired through the properties of breast milk. However, it is still necessary to conduct research on the complex network of molecules active in antimicrobial defense and protection present in breast milk. Understanding the antiviral properties of milk would have the final goal of improving the health status of the infants. For these reasons, the neutralizing activity of breast milk towards two types of respiratory viruses, SARS-CoV-2, an enveloped virus, and HAdV, a non-enveloped virus, will be tested in vitro in permissive cell lines. Furthermore, other aims of the study are the investigation of the milk antiviral defensive contributions of nonspecific compounds, such as fatty acids, and the *in vitro* analysis of the antiviral activity of oligosaccharide compounds, structurally identical to those contained in breast milk (HMO).

MATERIALS AND METHODS

2.1 Population enrollment and milk samples collection

The enrollment of the mothers was conducted from November 2023 to May 2024, at the neonatal intensive care unit and neonatal follow-up center of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico (Milan, Italy), a level III for neonatal care. To be eligible to participate in the current study, the mothers had to be over the age of 18, have good Italian comprehension to properly give written informed consent and be currently breastfeeding, even partially. Exclusion criteria, on the other hand, included not giving consent through the appropriate forms or being unfit to do so, not having a good comprehension of Italian, and not breastfeeding at the moment of the study. Each participating mother provided informed consent by signing the necessary documentation. The Ethics Committee "Milano 2" of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico authorized the research, which was carried out in compliance with the Declaration of Helsinki and hospital and municipal applicable government norms and laws. The total number of lactating mothers enrolled was 38, whose ages ranged from 23 to 42 years, with an average age of 34.5 years. Milk samples collection was scheduled on different postpartum days, ranging from 4 to 260 days, with an average of 28.7 days postpartum. Milk samples were divided in two groups: colostrum, for milk samples collected from 4 to 10 days postpartum and mature milk, for milk samples collected after 10 days postpartum and above. Colostrum group consists of 18 samples, where mature milk group consist of 20 samples.

The samples were collected either by manual expression or with the use of a breast pump device. All samples were personally provided by the mother. A total of 6 mL of breast milk sample was collected from each mother at a single time point. The collected milk sample was immediately placed into a sterile test tube and divided into two parts: one portion was sent directly to a laboratory for the measurement of fatty acid content, the other portion was stored in a -80°C freezer before being moved to the Molecular Virology laboratory (Department of Biomedical, Surgical and Dental

Sciences, University of Milan), which is a fully equipped biosafety-level-3 (BSL3) facility. Milk samples were kept frozen during the shipping and once at the Molecular Virology laboratory, were stored in a -80°C freezer till the subsequent analysis.

The present study includes also a set of milk samples collected by Istituto IRCCS Materno Infantile "Burlo Garofolo" in 2021, in the contest of human milk and SARS-CoV-2 vaccination research. A total of 36 milk samples were collected after the administration of two doses of vaccine, from 3 to 119 days post the second dose (mean of postvaccination days: 39.69). Each participating mother provided informed consent by signing the necessary documentation. The Ethics Committee of the IRCCS materno Infantile Burlo Garofolo authorized the research.

2.2 Measurement of fatty acid content

Measurement of fatty acid content was possible only for milk samples collected at the Policlinico of Milan. All the samples were analyzed within a maximum of one week from the collection. From each sample, 25 μL of mother's milk were extracted and transferred into a different vial and then methylated with 700 μL of hydrochloric acid in methanol (Sigma Aldrich, Saint Louis, MO, USA). After being heated at 90 °C for an hour and then cooled at 4 °C for ten minutes, potassium chloride (2 mL) and Hexane (400 μL) were added to the solution. All samples were first vortexed and then centrifuged at 3000 rpm for 10 min. Then, the upper layer was collected and transferred into a gas chromatography vial. The Shimadzu Nexis GC-2030 (Shimadzu, Japan) gas-chromatographer equipped with a 30m fused silica capillary column FAMEWAX Restek (Restek S.R.L., Cernusco sul Naviglio MI, Italy) was used for fatty acid profile evaluation. The gas chromatography results were analyzed using LabSolution software (ver. 5.97 SP1, Shimadzu Corporation, Kyoto, Japan). Single fatty acids were expressed as the relative percentage of total fatty acids.

2.3 Human milk sialylated oligosaccharides (HMOs)

A powder containing a mixture of some sialylated oligosaccharides structurally identical to those contained in human milk (HMO mixture of 3'-Sialyllactose, 6'-Sialyllactose, Disialyllacto-N-tetraose, LS, catalog number: OH165970, Biosynth) and another one containing one single specific oligosaccharide, the 6'-Sialyllactose

sodium salt (6'SL, catalog number: OS04398, Biosynth) were used to test *in vitro* their potential antiviral activity. HMOs are bioactive compounds, able to contribute to the innate immunity, to act as prebiotics for beneficial bacteria and, for these reasons, an increasing number of studies are investigating their antiviral activities *in vitro*[21].

2.4 Cell line models

2.4.1 Vero E6 cell line, maintenance and passaging

Vero E6 cell line (ATCC, catalog number: CRL-1586^{TM}) was the cell model used to perform part of the experiments of this study, like Median Tissue Culture Infectious Dose of SARS-CoV-2 ($TCID_{50}$) determination and SARS-CoV-2 microneutralization assay, because it had already showed susceptibility and permissivity to SARS-CoV-2 [63]. Vero E6 cell line is a mammalian immortalized cell line with an epithelial morphology, obtained by serial passaging of Vero cell line, which in turn was established in 1962 from the primary culture of epithelial cells derived from kidney of *Cercopithecus aethiops* (african green monkey)[6364]. Vero E6 cells express high levels of Angiotensin-converting enzyme 2 (ACE2) receptors, are unable to produce interferons and cells exhibit cytopathic effects (CPE), which are visible as cell rounding and detachment from the surface[63]. Vero E6 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with high glucose levels (*Euroclone*, Italy) supplemented with 10% Fetal Bovine Serum (FBS) (*Euroclone*, Italy), 1% penicillin (100 U/mL) and streptomycin (100 ug/mL) antibiotics and with L-glutamine amino acid (2 mM). Vero E6 cells grew in a monolayer adherent to the surface of ventilated flasks, kept in a cell incubator with a humidified atmosphere at 37°C in the presence of 5% CO_2. Cells were sub-cultured two times a week, upon reaching the cellular confluence.

For the purposes of the present study, cytopathic effects induced by SARS-CoV-2 infection were evaluated with an inverted optical microscope at six days post infection. At the same timepoint, not infected Vero E6 cells were used as control and showed an epithelial morphology, remaining adherent to the substrate.

2.4.2 A549 cell line, maintenance and passaging

A549 (ATCC, catalog number: CCL-185 ^{TM}) are type II alveolar epithelial cells, isolated from the lung tissue of a white, 58-year-old male, with lung cancer [65]. In the present study we chose A549 as the cellular model for median Tissue Culture Infectious Dose of Human Adenovirus ($TCID_{50}$) determination and for Human Adenovirus microneutralization assay (MNT). A549 are highly permissive cell to Adenovirus and are largely used in research concerning human adenovirus infection and response to it [27,66]. Cells exhibit cytopathic effects after 72 hours post infection, which are visible as cell rounding, detachment from the surface and interruption of the cell monolayer [67]. According to literature, in the present study, we evaluated CPE with an inverted optical microscope, three days post infection. Healthy A549 cells remain adherent to the substrate, in a continuous monolayer, whereas infected A549 cells show visible CPE.

A549 were maintained in Dulbecco's Modified Eagle Medium (DMEM) with high glucose levels (*Euroclone*, Italy) supplemented with 10% Fetal Bovine Serum (FBS) (*Euroclone*, Italy), 1% penicillin (100 U/mL) and streptomycin (100 ug/mL) antibiotics and with L-glutamine amino acid (2 mM). A549 were left adhere and grow in ventilated flasks and in a cell incubator with a humidified atmosphere at 37°C, containing 5% CO_2 and were sub-cultured two times a week, upon reaching the cellular confluence.

2.4.3 Calu-3 cell line, maintenance, passaging

Calu-3 (ATCC, catalog number: HTB-55^{TM}) epithelial cells are a human lung adenocarcinoma cells, derived from human bronchial submucosal glands, isolated in 1975 from the pleural effusion of a 25-year-old Caucasian male patient [68]. Calu-3 are applied in cancer research, toxicology research, drug development and as a human airway epithelial cell line model for studying respiratory virus like SARS-CoV and SARS-CoV-2 [63,69]. Their human origin allows to understand better human responses to human virus and to antiviral drugs[63], for these reasons, Calu-3 were used as the cell model for *in vitro* experiments regarding HMO's antiviral activity against SARS-CoV-2. Calu-3 are permissive to SARS-CoV-2, but the viral production is lower then in Vero E6 and no CPE are shown [63].

Calu-3 were maintained in Dulbecco's Modified Eagle Medium (DMEM) with high glucose levels (*Euroclone*, Italy) supplemented with 10% Fetal Bovine Serum (FBS) (*Euroclone*, Italy), 1% penicillin (100 U/mL) and streptomycin (100 ug/mL) antibiotics and with L-glutamine amino acid (2 mM). Calu-3 were left adhere and grow in ventilated flasks and in a cell incubator with a humidified atmosphere at 37°C, containing 5% CO_2 and were generally sub-cultured two times a week, upon reaching the cellular confluence.

2.5 50% Tissue Culture Infectious Dose ($TCID_{50}$) assay

The 50% Tissue Culture Infectious Dose ($TCID_{50}$) assay is a type of infectivity assay that measures viral particles capable of replicating in a particular cell type. It is an endpoint dilution assay in which serial dilutions of the virus stock are inoculated on the test units, represented by single wells of a multi-well plate, seeded with virus-susceptible and permissive cells. The result of the assay is the $TCID_{50}$ value, which is the dilution of the virus stock at which half of the test units are infected[51]. $TCID_{50}$ determination relies on the possibility of assessing the presence of virus induced CPE *in vitro* [70]. In this study, $TCID_{50}$ was performed to measure the infectivity of SARS-CoV-2 virions and Human Adenovirus virions previously isolated from cell cultures.

The following protocol was applied for SARS-CoV-2 $TCID_{50}$, using 96-well plate in vertical:

- dilute serially (1:10) SARS-CoV-2 virus stock across a first round-bottomed 96-well plate (rMW96-1): prior to serial dilutions, add 90 µL of complete medium to each well, then, add 10 µL of SARS-CoV-2 stock to the wells containing the medium in the first column (A) of the plate (reaching a final volume of 100 µL /well), dilute the virus across the multi-well plate from column A to column H by mixing 10 µL /well of the previous virus dilution with 90 µL /well of complete medium of the subsequent column, and repeat the same until obtaining serial 1:10 dilutions ranging from 1:10 to $1:10^{10}$. Test each dilution in quintuplicate. Dedicate 6 wells of the plate to the negative control (C-) for uninfected cells, containing 90 µL /well of complete medium only;

- perform an additional 1:2 dilution of the viral stock in a final volume of 50 µL/well: dispense 25 µL /well of complete medium into a second round-bottomed 96-well plate (rMW96-2) and transfer 25 µL of each dilution previously prepared in the rMW96-1 into the second round-bottomed 96-well plate (rMW96-2)
- transfer viral stock dilutions from the rMW96-2 to a flat-bottomed 96-well plate (fMW96), in which Vero E6 cells were seeded the day before with 100 µL/well of complete medium: prior to the transfer of viral serial dilutions, remove cell culture medium from each well of the fMW96, then transfer 30 µL of each SARS-CoV-2 stock dilution from the rMW96-2 to the fMW96 and do the same procedure for the C- wells;
- incubate the fMW96 in a cell incubator for 2 hours, at 37°C, with 5% CO_2, allowing virus replication;
- add 170 µL /well of complete medium, reaching a final volume of 200 µL/well, which is suitable to maintain the cells in culture for the following days;
- keep the fMW96 in the cell incubator at 37°C, with 5% CO_2, until CPE analysis that usually occurred five days post inoculum;
- perform the final analysis by detecting the presence or the absence of CPE in each well which represents the test unit, using an inverted optical microscope: if CPE can be seen, the test unit is scored positive, if not, the unit is scored negative; cells in the C- wells shouldn't show any cytopathic effect;

After the assessment of positive and negative scores to each test unit, perform $TCID_{50}$ calculation by applying the Reed and Muench formula, keeping into consideration that the titer to be found is that of an infective dose [70].

$$\text{Log}\, TCID50 \; =$$

$$\text{Log}\;(\text{Dilution showing mortality next above 50\%}) - (\text{Proportionate distance x Log Dilution factor})$$

- $\text{Proportionate distance} = \frac{\text{Mortality next above 50\%} - 50\%}{(\text{Mortality next above 50\%} - \text{Mortality next below 50\%})}$
- Dilution factor = 10

The following protocol was applied for Human Adenovirus (HAdV) $TCID_{50}$, using MW96 in vertical:

- dilute serially (1:10) HAdVs stock across a first round-bottomed 96-well plate (rMW96-1): prior to serial dilutions, add 90 µL of complete medium to each well, then, add 10 µL of HAdVs stock to the wells containing the medium in the first column (A) of the plate (reaching a final volume of 100 µL /well), dilute the virus across the multi-well plate from column A to column H by mixing 10 µL /well of the previous virus dilution with 90 µL /well of complete medium of the subsequent column, and repeat the same process for all the columns, until obtaining serial 1:10 dilutions ranging from 1:10 to $1:10^{10}$. Test each dilution in quintuplicate. Dedicate 6 wells of the plate to the negative control (C-) for uninfected cells, containing 90 µL /well of complete medium only;
- transfer viral stock dilutions from the rMW96 to a flat-bottomed 96-well plate (fMW96), in which A549 cells were seeded the day before with 100 µL /well of complete medium: prior to the transfer of viral serial dilutions, remove cell culture medium from each well of the fMW96, then transfer 30 µL of each virus stock dilution from the rMW96 to the fMW96 and do the same procedure for the C- wells;
- incubate the fMW96 in a cell incubator for 2 hours, at 37°C, with 5% CO_2, allowing virus replication;
- add 170 µL /well of complete medium, reaching a final volume of 200 µL/well, which is suitable to maintain the cells in culture for the following days;
- keep the fMW96 in the cell incubator at 37°C, with 5% CO_2, until CPE analysis that usually occurred after 72 hours post inoculum;
- perform the final analysis by detecting the presence or the absence of CPE in each well which represents the test unit, using an inverted optical microscope: if CPE can be seen the test unit is scored positive, if not, the unit is scored negative; cells in the C- wells shouldn't show any cytopathic effect;

Apply the Reed and Muench formula to get the $TCID_{50}$ titer.

2.6 Microneutralization test (MNT)

The microneutralization test is a gold standard technique in microbiology and virology for measure the capacity of a biological sample to neutralize infections [71]. In virology, the MNT allows the detection of neutralizing antibodies and antiviral activities of the biological sample, which can inhibit the virus replication in a virus-susceptible and permissive cell culture [51]. In diagnostic and research, neutralization assays are used to check the antibody-mediated protective immunity after infection or vaccination and usually are performed with serum samples [72]. MNT consist in the mixing of serial dilutions of the biological sample with a defined quantity of virus (expressed in $TCID_{50}$) and after a period of incubation, in which neutralizing antibodies eventually present in the biological sample can bind to the virions, sample/virus mixtures are inoculated into permissive culture cells and incubated. Then, the detection of virus-induced cytophatic effects (CPE) at the optical microscope, is used to determine if the viral infection occurred or not, and therefore the presence or absence of neutralizing activity[73]. Finally, with different mathematic methods, is possible to calculate the effective neutralization titer at 50% endpoint (NT_{50}), that means the highest sample dilution at which the 50% of the inoculated wells are protected from CPE. In the present study, the MNT was performed with milk samples, SARS-CoV-2 and HAdV viral particles in order to get the neutralization activity of milk samples. The efficiency of the MNT test was assessed testing some serum samples belonging to donor mothers. The NT_{50} for each milk/serum samples and for both viruses was calculated by means of Reed and Muench method keeping into consideration that the titer to be found is that of a protective biological sample (human milk/serum sample)

- For SARS-CoV-2 MNT the following protocol was applied, using MW96 in vertical:
- seed 15 000 Vero E6 cells/well in a flat-bottom 96 well plate (fMW96) the day before the experiment, left the cells adhere overnight in complete medium, at 37°C, in a humidified atmosphere containing 5% CO_2;
- thaw milk samples stored at -80°C and move 150 µL of each milk sample in tube;

- heat-inactivate each milk sample at 56°C for 40 minutes to inactivate complement proteins in the milk sample;
- prepare the first milk's dilution (1:2) by mixing 150 µL of the heat inactivated milk with 150 µL of complete cell medium in a tube, then add 50 µL of the 1:2 dilution in A2-A6 wells of a round-bottom 96 well plate (rMW96);
- dispense 25 µL /well of complete cell medium (avoiding the first column A2-A6), then prepare serial 1:2 dilution across the plate by mixing 25 µL of the previous milk dilution with 25 µL /well of complete medium. Prepare milk's dilutions ranging from 1:2 to 1:256 and prepare each dilution in quintuplicate. Dedicate 6 wells to the positive control (C+) and 6 wells to the negative control (C-), add 25 µL/well and 50 µL/well of complete medium respectively to C+ and to C- wells respectively;
- dilute SARS-CoV-2 virions stock with complete medium at the $200TCID_{50}$ (50% Tissue Culture Infectious Dose); add 25 uL/well of the diluted virus suspension to each well containing the diluted milk samples and to the 6 wells corresponding to C+;
- incubate milk samples mixed with virus for 1 hour, at 37°C, in a humidified atmosphere containing 5% CO_2;
- remove the cell culture medium from each well of the cell's plate (fMW96) and transfer 30 µL of each well's content of the rMW96 to the flat-bottom 96 well plate (fMW96); do the same procedure for the C+ and C- wells;
- add 170 µL of complete medium to each well, to reach a final volume of 200 µL /well
- keep the fMW96 in the incubator, at 37°C, in a humidified atmosphere containing 5% CO_2, for 6 days
- perform the final analysis by evaluating the presence or the absence of CPE in each well, using an inverted optical microscope

Calculate the 50% endpoint titer by applying the Reed and Muench formula:

$$\log_{10}(50\%\ Endpoint\ dilution) =$$

$$\log_{10}\ \text{Dilution showing mortality next above 50\%} - (\text{Proportionate distance x log10 Dilution factor})$$

- Proportionate distance $= \frac{\text{Mortality next above 50\% – 50\%}}{(\text{Mortality next above 50\% – Mortality next below 50\%})}$
- Dilution factor = 2

For Human Adenovirus MNT the same protocol of SARS-CoV-2 MNT was followed but with these different steps:

- seed 10 000 A549 cells/well in a flat-bottom 96 well plate (fMW96) the day before the experiment, left the cells adhere overnight in complete medium, at 37°C, 5% CO_2;
- keep the fMW96 in the incubator, at 37°C, in a humidified atmosphere containing 5% CO_2, for 4 days;

For SARS-CoV-2 MNT with serum samples, the same protocol of SARS-CoV-2 MNT with milk samples was followed. For Human Adenovirus MNT with serum samples, we repeat the test including serum at higher dilutions, above 1:256, because we didn't observe CPE considering dilutions from 1:2 to 1:256. The same protocol of Adenovirus MNT with milk samples was followed but with these different steps:

- Prepare dilutions from 1:2 to 1:32 in tubes with complete cell medium for each serum sample;
- Dispense 50 µL of the first dilution of serum sample (1:32) in the first column (A2-A6) of a round-bottom 96 well plate (rMW96), dispense 25 µL /well of complete cell medium (avoiding the first column A2-A6), then prepare serial 1:2 dilution across the plate by mixing 25 µL of the previous serum dilution with 25 µL/well of complete medium: prepare serum's dilutions ranging from 1:32 to 1:4096 in quintuplicate across the plate;

2.7 Plaque assay

In virology, a plaque assay is an experiment that is performed to measure and quantify the amount of viral infectious particles by quantifying the amount of plaques formed in a cell culture that is infected with serial dilutions of a pathogenic virus. In the present study, a plaque assay was performed to quantify the concentrations of the viral stock solutions. This concentration was used to calculate the viral dilution needed to infect Calu3 cells to test the antiviral activity of synthetic HMOs mixture and 6'SL. The plaque assay was performed by following a standard protocol. Vero E6 cells were plated on 6 well plates. A total of 750 000 cells were plated on each well in 2mL of complete medium. Afterwards, the plates were incubated for 24 hours at 37°C in a 5% humidified CO2 incubator. This time period allows the cells to attach to the support. After one night of growth, the supernatants were removed. A 10-fold serial dilution of the viral stock was performed in complete medium. Afterwards, each dilution was added in duplicate to the wells. Afterwards, the plates were incubated for 2 hours at 37°C in a 5% humidified CO2 incubator. The incubation allowed virions to attach to the ACE2 receptor of the Vero E6 cells. After the incubation period the inoculum was removed by discharging the supernatants. Next a washing step was performed by washing each well twice with 2mL of DPBS. After the washing, 3mL of 3% (m/V) agarose in complete DMEM solution was added to each well. Then, the plates were incubated for 48 hours at 37°C in a 5% humidified CO2 incubator. Next, 3mL of 3,7% (V/V) formaldehyde solution was added to each well. The plates were incubated for 1 hour at room temperature to fixate the cells. After the incubation, the medium and the agarose gel were removed from the wells and each well was washed twice with 2mL of DPBS. Afterwards, the cells were stained by adding 3 mL of the diluted methylene blue solution to each well. The plates were incubated at room temperature for 15 minutes and then the staining solution was discharged. Each well was washed three times with 2 mL of water and after the washing the plates were allowed to dry overnight. The next day a counting of the plaques was performed. Methylene Blue was able to stain the cells that are attached to the wells, the white spots are empty of cells and are determined as plaques. The plaques were counted as" plaque forming units" (PFU). The mean number of PFU was calculated for a dilution in which the plaques were easy countable. This number was multiplied by the dilution factor to calculate

the number of PFU per mL of viral suspension. These numbers were used to determine the multiplicity of infection. MOI refers to the amount of virions per cell during infection and was calculated by the use of the following formula: MOI=PFU/(number of cells)[74].

2.8 Antiviral activity test of HMOs mixture and 6'SL *in vitro*

The following protocol was applied to test the antiviral activity of HMOs mixture and of 6'SL, against SARS-CoV-2 in permissive cell line Calu-3, using a flat MW96 in vertical:

- seed 100 000 Calu-3 cells/well in a flat-bottom 96 well plate (fMW96) the day before the experiment, left the cells adhere overnight in complete medium, at 37°C, in a humidified atmosphere containing 5% CO_2. Use one single fMW96 to test both the compounds: divide the plate in order to have a first group of 7 columns for HMOs test (including a positive and negative control) and a second group of 9 columns for 6'SL test; test each concentration in triplicate;
- weigh HMOs mix and 6'SL in order to obtain the stock concentrations of HMOs= 10 mg/mL; vortex in order to obtain an homogeneous solution;
- prepare the initial concentration of HMOs mix and 6'SL to test on cells by diluting the stock with a dilution factor respectively of 20 and 10 in a tube; the initial concentration to test is 0,5 mg/mL and 1 mg/mL respectively;
- organize a round-bottom 96 well plate (rMW96) in order to have the same scheme of the cells, by dividing the plate in two parts, one dedicated to HMOs concentrations and one dedicated to 6'SL; then, create dilutions 1:4 of HMOs mix and 1:2 of 6'SL directly in the round-bottom 96 well plate in order to obtain the following concentrations to test on cells: 0.125 mg/mL, 0.031 mg/mL, 0.0078 mg/mL, 0.0019 mg/mL for HMOs mix and 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.0625 mg/mL, 0.031 mg/mL, 0.015 mg/mL for 6'SL. Dispense 150 µL of complete cell medium in each wells of the first group of column (c+ and c- included) and dispense 120 µL of complete cell medium in each wells of the second group of column

(c+ and c-) except for the first column that has to be filled with 200 µL/240 µL of the initial HMO/6'SL concentration solutions (0,5mg/ml – 1 mg/mL); then move 50 µL/120 µL from the first column to the second and to the subsequent columns (c+ and c- have to be filled only with cell medium)

- remove the cell culture medium from each well of the cell's plate (fMW96) and transfer 100 µL of each dilution to test from the rMW96 to fMW96 following the scheme of the plate (c+ and c- included);
- incubate for 24 hours, at 37°C, in a humidified atmosphere containing 5% CO_2;
- remove the cell culture medium containing HMOs and 6'SL from each well of the cell's plate (fMW96) and inoculate SARS-CoV-2, with MOI=0.1, 50 µL for each well (c+ included, c- excluded);
- incubate for 2 hours, at 37°C, in a humidified atmosphere containing 5% CO_2;
- remove the inoculum and add 200 µL of complete cell medium to each well and incubate for 48 hours, at 37°C, in a humidified atmosphere containing 5% CO_2;
- collect 10 µL of supernatant from each well and perform quantitative reverse transcription-polymerase chain reaction (qRT-PCR);

2.9 Real-Time PCR for SARS-CoV-2 N1 GENE

Quantitative Real-time Polymerase Chain Reaction (qPCR) is a gold standard molecular biology technique, mostly used in diagnostic and research laboratories, that allows the amplification and quantification of specific sequence of nucleic acid in real-time[75]. In the present study, a variant of qPCR, called quantitative reverse transcritption-polymerase chain reaction (qRT-PCR) was performed for the detection and quantification of SARS-CoV-2 nucleic acid in the supernatants of cell cultures, after the pre-treatment of cells with HMOs mixture and 6'SL, in order to get a value of percentage of replication of the virus and to see possible antiviral effects due to the treatment. In qRT-PCR assays, RNA is first transcribed into complementary DNA

(cDNA). The cDNA is then used as the template for the quantitative real-time PCR reaction (qPCR). The quantification of the product occurs through the fluorescence measured for each qPCR cycle. The amount of fluorescence is directly proportional to the amount of amplified product[76]. PCR can be split into 3 phases that occur in each cycle of amplification. The first phase is denaturation: here the target DNA is separated into individual DNA filaments at temperatures of 94°C for about 30 to 60 seconds. In the second phase, the primers, added to the sample mix, hybridize to the single DNA fragments in presence of the target. This phase of hybridization occurs at temperatures between 50°C to 60 °C. Extension of the new strand is the third phase in which at an ideal temperature (65-72°C), the Taq polymerase enzyme attaches nucleotides to the primers and copies a complementary strand of the template. Repetition of these phases results in exponential amplification of DNA target. There are two kinds of Real-Time PCR, one-step PCR and two-step PCR. In a two-step, real-time PCR reverse transcription and amplification are done separately. In this study, AgPath-ID™ One-Step RT-PCR kit (ThermoFisherScientific, USA) was used. For fluorescence measurements, TaqMan probes were used. These types of probes consist of oligonucleotide with a 5′ modified end with a reporter fluorophore and a 3′ modified end with a quencher. TaqMan chemistry utilizes the exonuclease activity of the Taq polymerase that breaks the reporter from proximity with the quencher allowing fluorescence and quantification of the target sequences.

In this study, a total volume of 20 μL of the reaction mix and 5 μL of sample RNA was used. The reaction mix was composed of 2x RT-PCR buffer, 25x RT-PCR enzyme, nuclease-free water, a final concentration of 400 nM of each primer (2019-nCoV_N1-F and 2019-nCoV_N1_R) and a final concentration of 100 nM of the probe (2019-nCoV_N1-P). Primers and probe sequences used are shown in figure 21, while constituents of the reaction mix are shown in figure 22. Absolute quantification of SARS-CoV-2 viral copies was done by creating a standard curve using a serially diluted plasmid pEX-A128-nCoV_all (Eurofins, Luxemburg), containing part of the SARS-CoV-2 genome (3x107–3x101 copies/μL). Samples were analyzed in triplicate, and negative control, consisting of a mix supplemented of water, was added. The limit of viral genome detection was evaluated as 3 copies/reaction. The thermal profile was as follow: a holding stage at 45 °C for 30 minutes allowed the action of reverse

transcriptase enzyme; 10 minutes at 95 °C to inactivate the reverse transcriptase and initiation of denaturation; 15 sec of DNA denaturation and 30 seconds at 55 °C that allows annealing of primers and incorporation of nucleotides by polymerase for a total of 45 cycles.

Primers	Position*	Sequences (5'-3')
2019-nCoV_N1-F	28287-28306	GAC CCC AAA ATC AGC GAA AT
2019-nCoV_N1-R	28335-28358	TCT GGT TAC TGC CAG TTG AAT CTG
2019-nCoV_N1-P	28309-28332	FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1
sgLeadSARSCoV2		CGATCTCTTGTAGATCTGTTCTC

*Nucleotide positions are referred to SARS-CoV-2 isolate Wuhan-Hu-1 complete genome (NC_045512.2)

Figure 21. Primer sequences for SARS-CoV-2 genomic real-time PCR assay.

Reagents	Initial concentration	Final concentration	uL x1 sample
2x RT-PCR Buffer	2x	1x	12.5
2019-nCoV N1 Fw	100 uM	400 nM	0.1
2019-nCoV N1 Rev	100 uM	400 nM	0.1
2019-nCoV N1 Probe	100 uM	100 nM	0.025
25x RT-PCR Enzyme Mix	25x	1x	1.0
Nuclease-free water			6.27
	Master Mix		**20.0**
	RNA		**5.0**

Table 1. Composition of the reaction mix for SARS-CoV-2 genomic N1 gene.

2.10 Human IgA ELISA

For the quantitative measurement of IgA in milk's serum, Human IgA SimpleStep ELISA kit (ab196263) was performed twice. The enzyme-linked immunosorbent assay (ELISA) is a type of enzymatic immunoassay in which the detection of antigen-antibody interactions is possible thanks to the presence of an antibody tagged with an enzyme which can cleave a chromogenic substrate generating a colored product[51]. In general, this assay requires the presence of antibodies or antigens attached onto the surface of a plate, but there are different types of ELISA assay. Human IgA ELISA kit is a Sandwich type ELISA in which a capture antibody specific for the target analyte or antigen is coated onto the surface of a microtiter plate and when the test sample is applied, and any target analyte (A) present, bound to the capture antibody; then a second antibody (known as the detection antibody) is applied, and binds the target

analyte that remains bound by the capture antibody. A secondary antibody conjugate is added and binds to the detection antibody reacting with a substrate to produce a signal that is detectable by specific reader instrument such as Synergy-reader (fig.21)[77]. In quantitative ELISA assay, the optical density (OD) of the output signal from every sample is compared to a standard curve that is included in every ELISA run. A standard curve is typically a serial dilution of a standard preparation of the target analyte, of which the starting concentration is known.

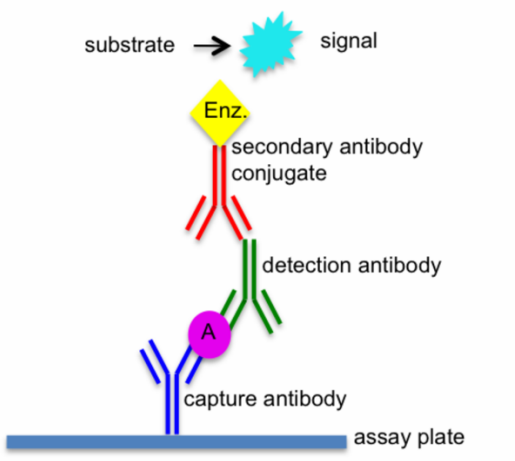


Figure 22. Sandwich ELISA format (Nordic Biosite).

Prior to performing the assay, we apply the following protocol in order to de-fat milk samples:

- centrifuge 200 µL of each milk sample at 500g for 15 minutes at 4°C and collect the aqueous fraction;
- centrifuge two times the aqueous fraction at 3000g for 15 minutes at 4°C and collect the final aqueous fraction;
- store un-diluted de-fatted milk samples at 4°C;
- dilute each milk sample 1:20000 into Sample Diluent in tubes;

Human IgA SimpleStep ELISA assay was performed following the manufacturer's instructions:

- equilibrate all reagents to room temperature prior to use;
- prepare the Wash Buffer PT(1X) by diluting 8.7 mL of pure Wash Buffer PT (10X) in 78.3 mL of deionized water; mix thoroughly and gently;
- prepare the Antibody Cocktail by diluting 410 µL of both capture and detector antibodies in 3280 mL of Antibody diluent; mix thoroughly and gently;

- prepare the set of standards:
  - reconstitute the IgA standard sample by adding the volume of Sample Diluent NS indicated on the protein vial label, hold at room temperature for 10 minutes, mix thoroughly and gently; this is the 100 ng/mL Stock Standard Solution
  - label eight tubes (standards 1-8), add 150 µL of Sample diluent NS into tubes
  - transfer 150 µL from the Stock Standard solution into the first tube and then transfer 150 µL from the first tube to the second and to the same for the subsequent tubes (dilution 1:2) except for the tube 8 that has to be filled only with Sample diluent; obtain the following concentrations: 100 ng/mL Stock Standard Solution, 50 ng/mL Standard 1, 25 ng/mL Standard 2, 12.5 ng/mL, 6.25 ng/mL, 3.13 ng/mL, 1.56 ng/mL; tube 8 will be the Blank Control
- remove excess microplate strips from the plate frame
- add 50 µL of all sample and standard to appropriate wells
- add 50 µL of the Antibody Cocktail to each well
- seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm
- wash each well with 3 x 350 uLwash buffer 1X, particularly after the last wash invert the plate and tap gently against clean paper towels to remove excess liquid
- add 100 µL of TMB Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm (optimal incubation time may vary between 5 and 20 minutes)
- add 100 µL of Stop Solution to each well, shake plate on a plate shaker for 1 minute to mix
- record the OD at 450 nm and proceed with calculation (performed by Gene5 software): calculate the average absorbance value for the blank control standards, subtract the average blank control standard absorbance value from all other absorbance values, create a standard curve by plotting the average blank control subtracted absorbance value for each standard concentration

against the target protein concentration of the standard, use graphing software to draw the best smooth curve through these points to construct the standard curve; determine the concentration of the target protein in the sample by interpolating the blank control subtracted absorbance values against the standard curve. Multiply the resulting value by the appropriate sample dilution factor, to obtain the concentration of target protein in the sample.

2.11 Human Anti-SARS-CoV-2 IgG Antibody to Spike protein S1 Quantitative TITRATION ELISA, GENLISA

For the quantitative measurement of Anti-SARS-CoV-2 IgG antibody in milk' serum GENLISA assay that employs sandwich ELISA technique was performed.

Prior to performing the assay, the following protocol in order to de-fat milk samples was applied:

- centrifuge 200 uL of each milk sample at 500g for 15 minutes at 4°C and collect the aqueous fraction;
- centrifuge two times the aqueous fraction at 3000g for 15 minutes at 4°C and collect the final aqueous fraction;
- store un-diluted de-fatted milk samples at 4°C;
- dilute each milk sample 1:1000 in tubes using Sample diluent(1X);

GENLISA ELISA assay was performed following the manufacturer's instructions:

- bring all reagents to room temperature before use;
- make Wash Buffer (1X) by diluting 25 mL of Wash Buffer in 475 mL of DI water;
- prepare Standards: reconstitute the concentrated Standard lyophilized vial with 1mL of Standard Diluent to obtain a concentration of 5000ng/mL, keep the vial for 15 min with gentle agitation before making further dilutions; dilute 200 µL of original Standard (5000 ng/mL) with 800 µL of Standard Diluent to generate a 1000 ng/mL Standard solution; prepare further Standards by serially diluting the Standard Solution 1:2 in tubes (1-7) and making the following concentrations: std 7 is the first standard solution= 1000 ng/mL; std 6= 500 ng/mL; std 5= 250 ng/mL; std 4= 125 ng/mL; std 3=

62.5 ng/mL; std 2= 31.25 ng/mL; std 1= 15.625 ng/mL; use Standard Diluent as the Zero Standard (blank);

- pipette 100 µL of Standards and diluted samples to the respective wells;
- seal plate and incubate for 1 hour at Room temperature;
- wash plate 4 times with Wash Buffer (1X) and blot residual buffer by firmly tapping plate upside down on absorbent paper;
- add 100 µL of Goat Anti-Human IgG:HRP Conjugate to each well;
- seal plate and incubate for 1 hour at Room temperature;
- wash plate 4 times with Wash Buffer (1X) and tap to remove any liquid;
- pipette 100 µL of TMB Substrate solution in all wells;
- incubate in the dark for 15 minutes at Room temperature;
- stop reaction by adding 100 µL of Stop Solution to each well;
- read absorbance at 450 nm within 30 minutes of stopping reaction and calculate results (Gene5 software): using standards graph paper, plot the absorbance value (450 nm) of each standard on the y-axis versus the corresponding concentration of the standards on the x-axis, determine the IgG concentrations through interpolation with the standards curve (polynomial regression or cubic spline curve-fit for automated results); multiply each sample concentrations by the appropriate dilution factor (1000);

GENLISA assay was repeated following the same protocol, but diluting each sample 1:100 because many sample at dilution 1:1000 had absorbance value below the first standard detection[78].

2.12 Statistical analysis

Statistical analysis was performed with GraphPad Prism 10 (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com). Categorical and numerical variables were described using medians and minimum/maximum values. Median Anti-SARS-CoV-2 IgG and median NT_{50} values were compared using Mann–Whitney U test as appropriate. Pearson correlation analysis was used to analyze correlation between immunoglobulin levels, fatty acids and paired NT_{50} values.

RESULTS

3.1 Population and sample features

Breast milk samples were collected in two different Institutions, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico Milan (POLI) and IRCCS Materno Infantile Burlo Garofolo Trieste (TS). Samples were collected at POLI from November 2023 to May 2024. Table 1 summarized demographic data of the mothers and features of the samples.

	POLI	**TS**
Samples number	38	36
Mothers age		n.a
Mean	34.5	n.a
Min	23	n.a
Max	42	n.a
Vaccination status	n.a	
Mean days post second dose vaccine	n.a	39,68
Min	n.a	3
Max	n.a	119
Collection days postpartum		>30
Mean	28.7	>30
Min	4	>30
Max	260	

Table 2. Population and milk samples characteristic.

Based on the described characteristics, milk samples collected at POLI were divided in two groups: 18 colostrum samples, collected from 4 to 10 days postpartum, and 20 mature milk samples, collected from 10 to 260 days postpartum. Samples collected at TS were considered as mature milk, since they were collected more then 30 days postpartum.

3.2 Breastmilk IgA

Level of total IgA was determined by quantitative simplestep ELISA assay and the results are shown in figure 24.

POLI Samples:

In colostrum group, IgA were present in 18/18 (100%) samples at dilution 1:20000, with a median value of 950000 ng/mL and a range of 287860-950000 ng/mL.

In mature milk group, IgA were present in 20/20 (100%) samples at dilution 1:20000, with a median value of 950000 ng/mL and a range of 245180-972050 ng/mL.

TS Samples:

IgA level was determined in 32/36 (88%) samples because the lack of samples for 4 of them. IgA were present in 32/32 (100%) samples, with a median value of 950000 ng/mL and a range of 271990-998930 ng/mL.

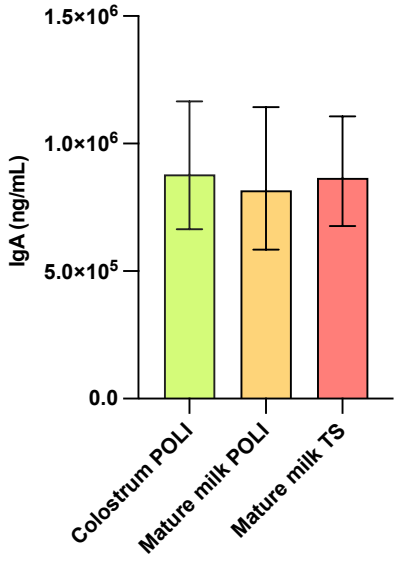


Figure 23. Comparison of total IgA levels in the human milk collected at POLI (colostrum and mature milk) and at TS (mature milk). The bars represent the median, and the error bars the standard deviation (SD).

3.3 Breastmilk Anti SARS-CoV-2 IgG

Level of Anti SARS-CoV-2 IgG was determined by quantitative GENLISA test, using a dilution of 1:1000, or, when necessary, a dilution of 1:100. Results are shown in figure 25. Briefly:

POLI Samples

In colostrum group, Anti SARS-CoV-2 IgG were present in 12/18 (66,6%) samples, with a median value of 2425 ng/mL and a range of 1605,75-41451 ng/mL.

In mature milk group, Anti SARS-CoV-2 IgG were present in 13/20 (65%) samples, with a median value of 2063 ng/mL and a range of 1598,65-29767,45 ng/mL.

TS Samples

Anti SARS-CoV-2 IgG level was determined in 32/36 (88%) samples because the lack of samples for 4 of them. Anti SARS-CoV-2 IgG were present in 27/32 (84.3%) samples, with a median value of 6925 ng/mL and a range of 1821,75-60140 ng/mL. Significant difference was found in median IgG value between POLI Mature milk samples and TS Mature milk samples (p=0.0044).

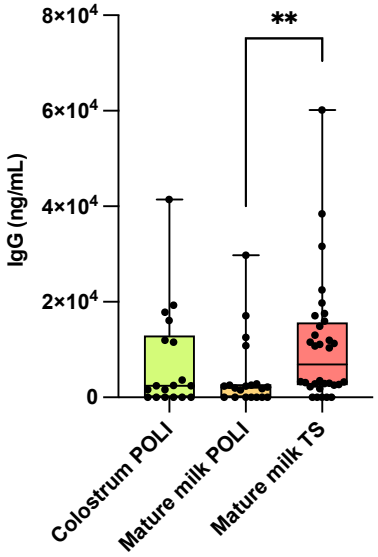


*Figure 24. Comparison of total Anti SARS-CoV-2 IgG levels in the human milk collected at POLI (colostrum and mature milk) and at TS (mature milk). The boxes extend from the 25th to the 75th percentiles, plots whiskers down to the minimum and up to the maximum value, each individual value is shown on the graph and the middle line of each box shows medians. **p=0.0044 (statistical test: Mann-Whitney U test).*

3.4 Breastmilk SARS-CoV-2 Neutralizing Activity

The neutralizing activity of breast milk samples against SARS-CoV-2 was quantified using NT_{50} values (fig.26).

POLI Samples

In colostrum group, a total of 13/18 (72%) samples showed neutralizing activity against SARS-CoV-2 B.1 lineage, with a median NT_{50} value of 1:5,42 and a range of 5,19-41,50.

In mature milk group, we calculate SARS-CoV-2 NT_{50} for 19 samples; one of them was not analyzable because of bacterial contamination. A total of 13/19 (68,4%)

samples showed neutralizing activity against SARS-CoV-2 B.1 lineage, with a median NT_{50} value of 1:2,83 and a range of 2,24-20,75.

TS Samples

Among TS milk samples, we were able to calculate SARS-CoV-2 NT_{50} titer for 23/36 (64%) samples, because 13 samples showed high bacterial contamination, which not allow the analysis of MNT test. A total of 10/23 (43,5%) showed neutralizing activity against SARS-CoV-2 B.1 lineage, with a median value of 0 and a range of 2-19.

A significant difference was found in median value NT_{50} against SARS-CoV-2 between POLI Colostrum and TS Milk groups (p=0.0466).

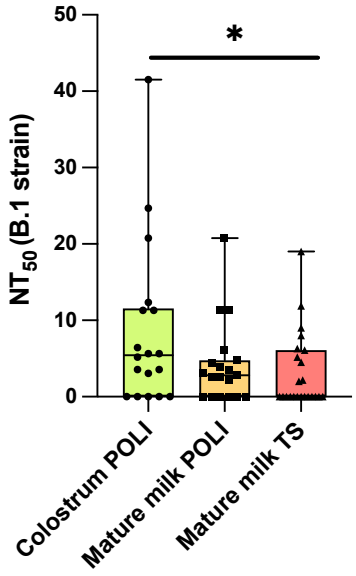


*Figure 25. NT_{50} values against SARS-CoV-2 B.1 lineage in Colostrum, Mature Milk, Trieste Milk. The boxes extend from the 25th to the 75th percentiles, plots whiskers down to the minimum and up to the maximum value, each individual value is shown on the graph and the middle line of each box shows medians. *p=0.0466 (statistical test: Mann-Whitney U test).*

3.5 Pearson correlation and linear regression between NT50 titers and IgG Anti-SARS-CoV-2

To examine the relation between NT_{50} SARS-CoV-2 titers and Anti-SARS-CoV-2 IgG, Pearson correlation and linear regression analysis, were conducted.

POLI Samples

In Pearson correlation analysis of POLI colostrum group, a Pearson coefficient of 0,35 was found, indicating a moderate positive correlation between NT_{50} and Anti SARS-

CoV-2 IgG (fig.27). Linear regression analysis showed an R^2 value of 0,12 and a p-value of 0,14.

In Pearson correlation analysis of POLI mature milk samples, a Pearson coefficient of 0,24 was found, indicating a weak positive correlation between NT_{50} and Anti SARS-CoV-2 IgG (fig.28). Linear regression analysis showed an R^2 value of 0,06 and a p-value of 0,30.

TS Samples

In Pearson correlation analysis of TS mature milk samples, a Pearson coefficient of 0,62 was found, indicating a strong positive correlation between NT_{50} titers and Anti SARS-CoV-2 IgG (fig.29). Linear regression analysis showed an R^2 value of 0,38 and a p-value of 0,003, indicating a statistically significant correlation.

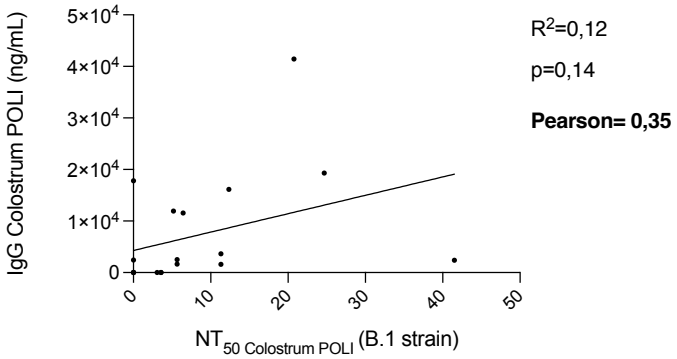


Figure 26. Pearson's correlation between IgG concentration and NT_{50} titers in POLI colostrum group of milk samples.

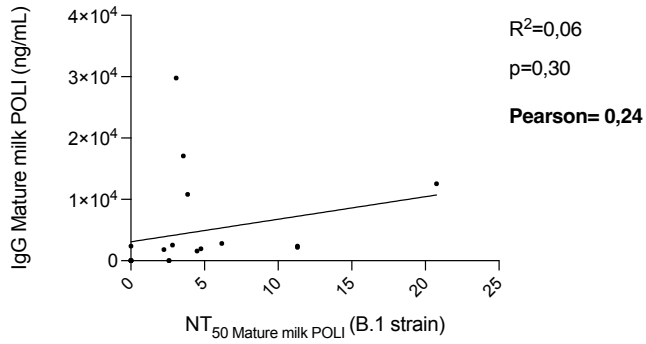


Figure 27. Pearson's correlation between IgG concentration and NT_{50} titers in POLI mature milk group.

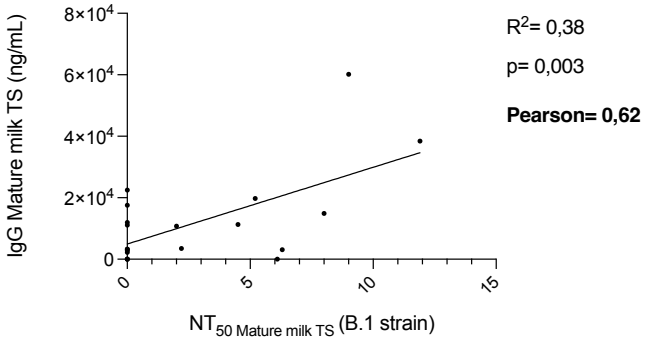


Figure 28. Pearson's correlation between IgG concentration and NT_{50} titers in TS mature milk group.

3.6 Milk samples IgG negative with neutralizing activity

A group of milk samples from POLI colostrum, POLI mature milk and TS mature milk groups, showed neutralizing activity although the Anti SARS-CoV-2 IgG were absent. Data regarding this specific group are summarized in table 2.

IDENTIFICATION DATA	POSTPARTUMDAYS	IgA mean	IgG mean	NT_{50} SARS CoV 2	MILK ANLYSIS DATA														
					16:0	16:1n7	18:0	18:1n9	18:1n7	18:2n6	18:3n3	20:3n9	20:3n6	20:4n6	20:5n3	22:0	22:5n3	24:0	22:6n3	24:1
#POLI 1	6	950000	0	3,56	23,73	2,66	4,64	44,11	3,14	18,77	0,50	0,04	0,58	0,76	0,30	0,14	0,09	0,08	0,30	0,12
#POLI 2	10	950000	0	3,08	18,93	0,90	5,46	50,31	1,20	20,37	0,79	0,08	0,67	0,45	0,03	0,05	0,09	0,12	0,33	0,16
#POLI 3	4	950000	0	3,56	23,41	1,67	6,27	45,79	1,70	18,67	0,55	0,02	0,37	0,80	0,01	0,06	0,11	0,08	0,32	0,09
#POLI 4	16	950000	0	2,59	27,69	2,03	7,38	46,24	2,34	11,74	0,54	0,08	0,44	0,68	0,08	0,20	0,10	0,06	0,24	0,11
#POLI 5	260	883980	0	2,59	23,78	1,73	6,73	43,59	2,08	20,08	0,61	0,04	0,26	0,46	0,01	0,07	0,12	0,07	0,30	0,05
#TRIESTE 1	n.a	647030	0	6,1	n.a															

Table 2. Three milk samples from POLI colostrum group, two samples from POLI mature milk group and one sample from TS mature milk group, Anti SARS-CoV-2 IgG negative, showed neutralizing activity. IgA concentration, NT_{50} value and Fatty acids content are indicated.

3.7 Fatty acids content of milk samples

The families of fatty acids analyzed in our study, were chosen considering the more already investigated antimicrobial properties. We focused on the profile of long chain polyunsaturated fatty acids (LCPUFAs), opting for the most abundant, the clinically most relevant and studied, and the most representative entities of each family. Fatty acids content in the 38 POLI Samples (colostrum and mature milk groups together) yielded the results shown in table n.2. Data are presented as mean percentage (±SD) and range.

FAs	Mean % of Total Fas (with SD) by Weight	Range
Palmitic (C16:0)	25,34±0,10	26,51-33,99
Palmitoleic (C16:1 n-7)	1,80±0,57	2,08-3,32
Stearic (C18:0)	6,45±1,93	4,28-9,16
Oleic (C18:1 n-9)	45,28±4,78	42,66-52,06
Vaccenic (C18:1 n-7)	3,25±0,99	0,34-15,96
Eicosatrienoic (C20:3 n-9)	0,08±0,04	0,01-0,57
Dihomo-γ-linolenic (C20:3 n-6)	0,58±0,54	0,37-1,33
Linoleic (C18:2 n-6)	14,72±3,28	11,07-28,67
α-linoleic (C18:3 n-3)	0,77±0,76	0,66-3,41
Arachidonic (C20:4 n-6)	0,66±0,38	0,54-1,08
Eicosapentanoic (C20:5 n-3)	0,05±0,17	0,03-0,3
Docosapentanoic (C22:5 n-3)	0,16±0,38	0,15-0,69
Docosahexanoic (C22:6 n-3)	0,50±0,82	0,55-1,77
Behenic (C22:0)	0,10±0,02	0,03-0,27
Lignoceric (C24:0)	0,11±0,03	0,07-0,22
Nervonic (C24:1)	0,13±0,01	0,06-0,45

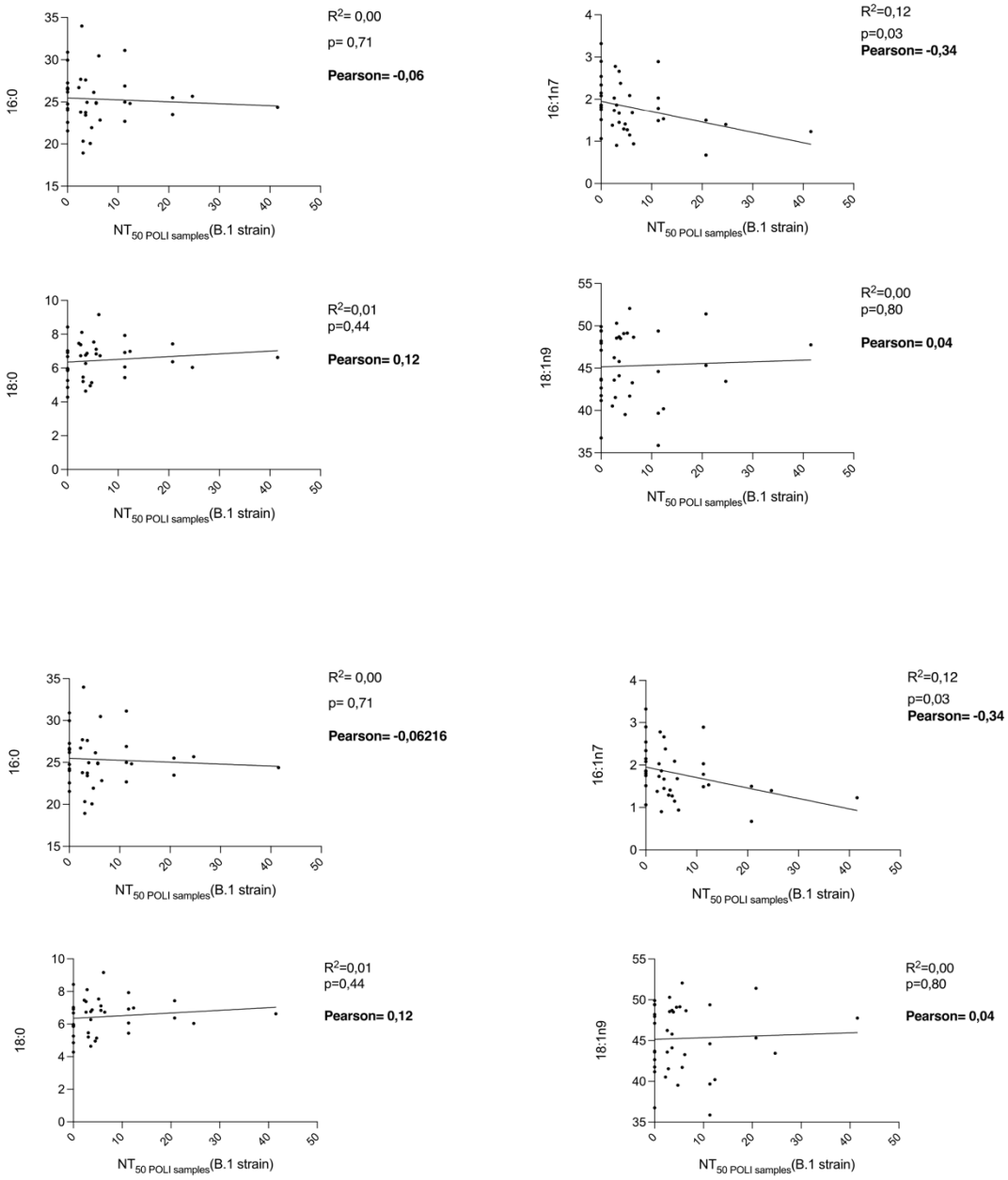
Table 3 FAs data.

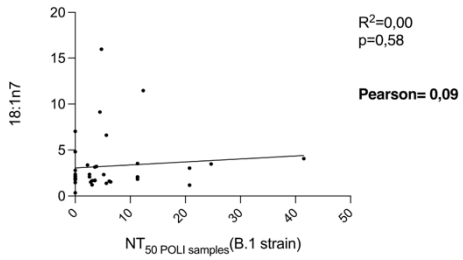
No significant difference in the mean LCPUFAs concentrations were found between POLI colostrum group and POLI mature milk group, except for Lignoceric (C24:0) and Nervonic (C24:1) FAs. Consequently, the analysis were performed considering the milk' samples, without any stratification, except for Lignoceric (C24:0) and Nervonic (C24:1) FAs.

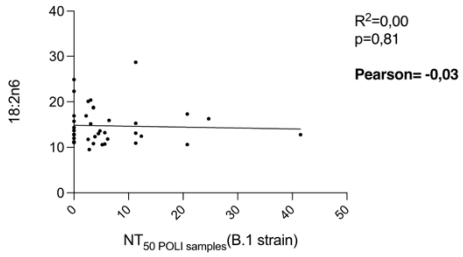
Among the various LCPUFAs that were analyzed, Nervonic acid (C24:1) and Lignoceric acid (C24:0) showed a statistically significant correlation, with a Pearson coefficient, respectively, of 0,36 and 0,30 when analyzed without group stratification (fig.30). Likewise, a tendency for correlation can be seen for Lignoceric (C24:0) FA both in POLI Colostrum group and POLI Mature milk group, with a Pearson coefficient of, respectively, 0,21 and 0,25; also Nervonic (C24:1) FA showed a tendency for correlation both in POLI Colostrum group and POLI Mature milk group, with Pearson coefficient, respectively, of 0,27 and 0,23 (fig. 31, fig. 32).

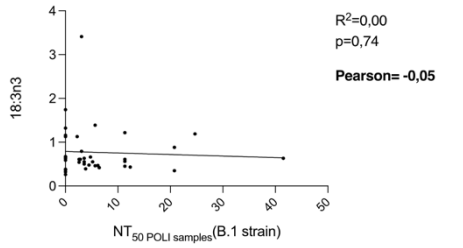
Other FAs showed tendency for correlation: Arachidonic acid (C20:4n6) has a Pearson coefficient of 0,24, Behenic acid (C22:0) has a Pearson coefficient of 0,21 and Stearic acid (C18:0) has a Pearson coefficient of 0,12.

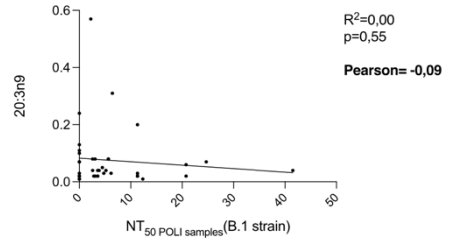
On the contrary, Palmitoleic acid (16:1n7) showed a moderate negative correlation, with a Pearson coefficient of -0,34 (fig.30).

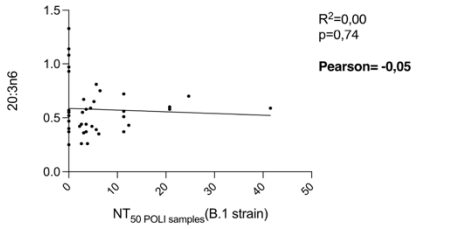


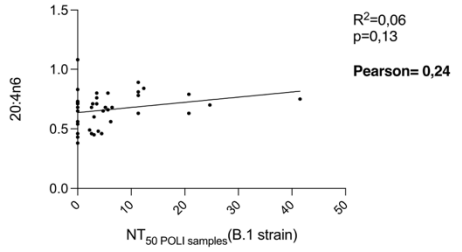

18:1n7
R^2=0,00
p=0,58
Pearson= 0,09
$NT_{50\ POLI\ samples}$(B.1 strain)

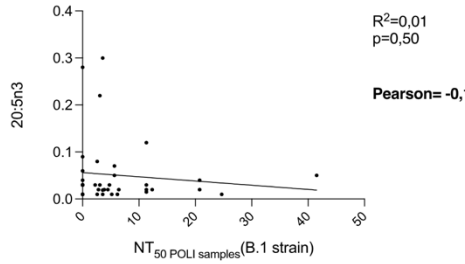

18:2n6
R^2=0,00
p=0,81
Pearson= -0,03
$NT_{50\ POLI\ samples}$(B.1 strain)

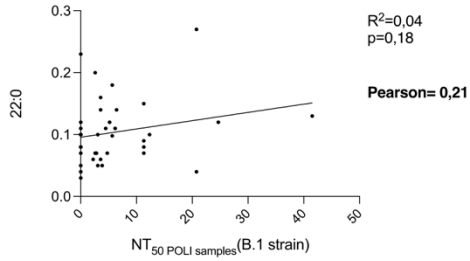

18:3n3
R^2=0,00
p=0,74
Pearson= -0,05
$NT_{50\ POLI\ samples}$(B.1 strain)


20:3n9
R^2=0,00
p=0,55
Pearson= -0,09
$NT_{50\ POLI\ samples}$(B.1 strain)


20:3n6
R^2=0,00
p=0,74
Pearson= -0,05
$NT_{50\ POLI\ samples}$(B.1 strain)


20:4n6
R^2=0,06
p=0,13
Pearson= 0,24
$NT_{50\ POLI\ samples}$(B.1 strain)


20:5n3
R^2=0,01
p=0,50
Pearson= -0,11
$NT_{50\ POLI\ samples}$(B.1 strain)


22:0
R^2=0,04
p=0,18
Pearson= 0,21
$NT_{50\ POLI\ samples}$(B.1 strain)

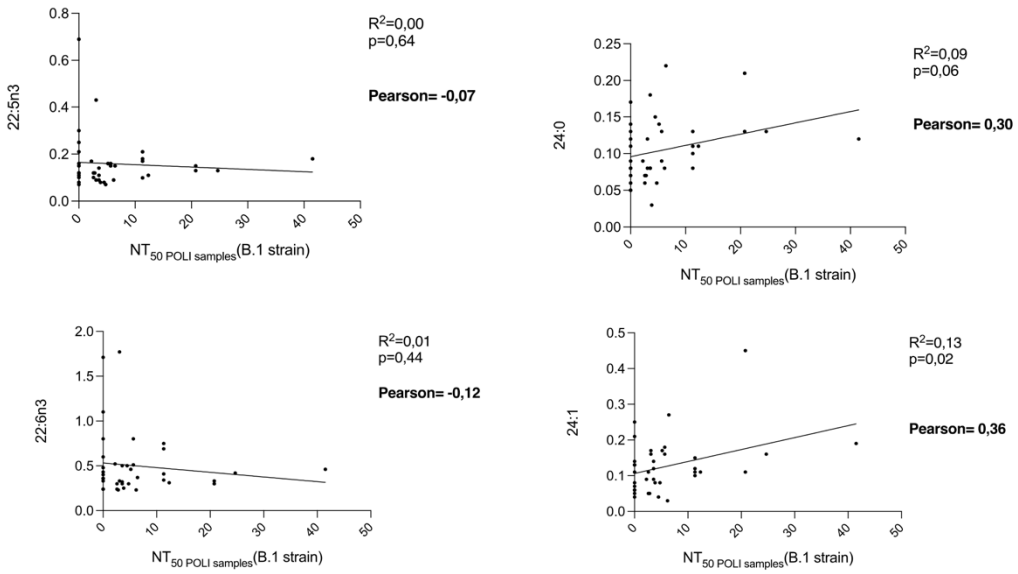


Figure 30. Pearson correlation analysis between NT_{50} POLI samples (Colostrum and Mature milk) and Fas.

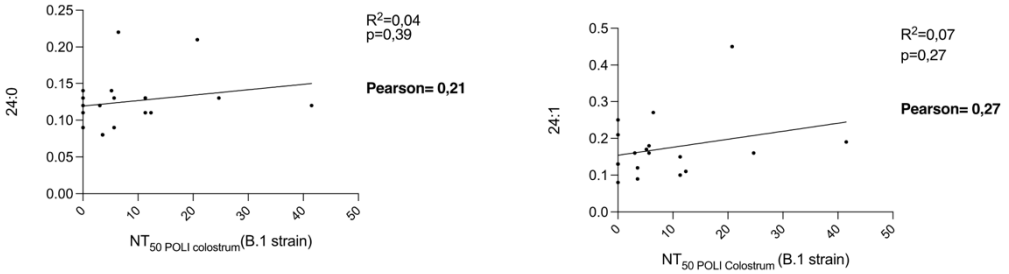


Figure 31. Pearson correlation analysis NT_{50} POLI Colostrum and Fas.

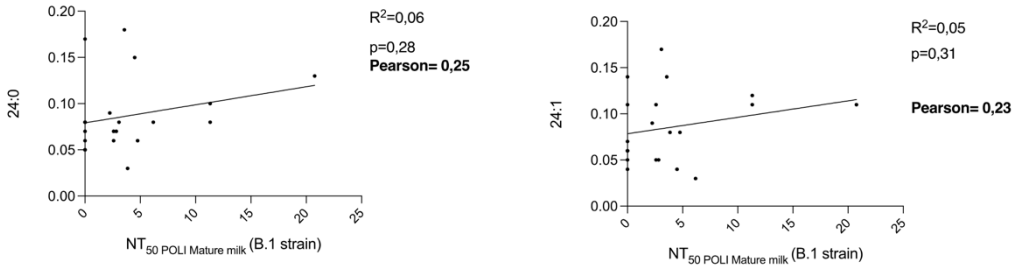


Figure 32. Pearson correlation analysis NT_{50} POLI Mature milk and Fas.

3.8 Breastmilk HAdV Neutralizing Activity

The neutralizing activity of breast milk samples against HAdV (C.1) was quantified using NT_{50} values.

POLI

No neutralizing activity against Adenovirus was observed in both colostrum and mature milk groups (data not shown).

TS Samples

Among TS milk samples, we were able to calculate HAdV NT_{50} titre for 8/36 (22%) samples, because 28 samples showed high bacterial contamination, which not allow the analysis of MNT test. A total of 2/8 (25%) showed neutralizing activity against HAdV, with a mean value of 1:33,94 and a range of 22,63-45,25.

3.9 In vitro evaluation of HMOs mixture and 6'SL

The antiviral activity of HMOs mixture and of 6'SL, against SARS-CoV-2, was tested in permissive cell line (Calu-3). Upon SARS-CoV-2 infection, viral replication was quantified in the culture medium 48 h post infection by qRT-PCR, evaluating the activity of either HMOs mixture or 6'SL. HMO showed an increasing antiviral activity (20-50%) when used at the concentration 0.002-0.125 ng/mL.

On the contrary, 6'SL by itself did not show specific and significant anti-SARS-CoV-2 activity at the tested concentration (fig.33).

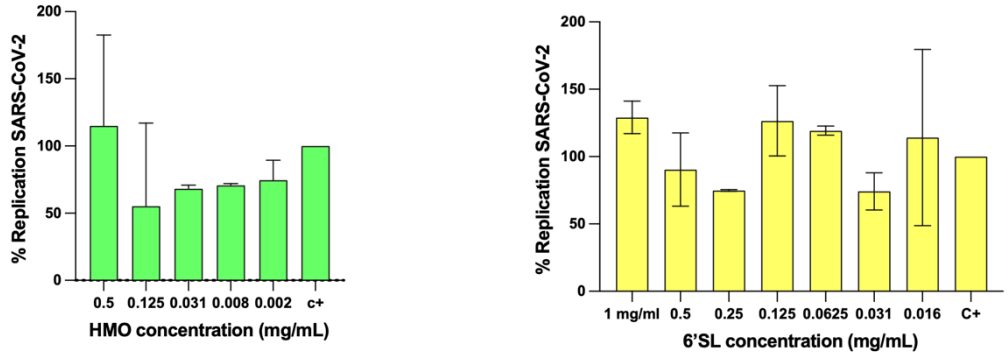


Figure 33. Antiviral effect (expressed as % of viral replication) of HMO and 6'SL against SARS-CoV-2 measured by qRT-PCR assay. The percentage of replication in Calu-3 cells by the compounds was expressed as the change of the viral load in the culture medium compared to the infected untreated cells (100%). The bars represent the mean value of two independent experiments and the error bars the standard deviation (SD).

DISCUSSION

The objectives of our study were to validate the neutralizing capacity of human milk against an enveloped virus, SARS-CoV-2, and a non-enveloped virus, HAdV, to explore the roles of the specific protective compounds such as Immunoglobulins A-G, and to analyze the antiviral contribution of nonspecifc protective compounds. As expected ([13]), all samples contain a very high levels of total IgA, being the most abundant family of Immunoglobulin in the mammary secretion. We performed a general quantitative ELISA assay for detection of global IgA in order to have a further parameter regarding adaptive immunity, that give us the chance to discriminate better between adaptive and innate antiviral activity of breast milk. In most of the POLI milk samples, and in half of the TS milk samples, the IgA concentrations were above the highest limit of ELISA detection. Unfortunately, we did not have the possibility to discriminate among anti-virus specific IgA; this could have given us the opportunity to draw more specific conclusions regarding their role in the antiviral activity.

With regard to the anti-SARS-CoV-2 IgG, they resulted more abundant in the milk samples collected from the TS cohort, which comprised mothers subjected to COVID-19 vaccination, in the previous 40 days, than in the milk samples collected from the POLI cohort. It is well known that short vaccine intervals (3 to 6 weeks) resulted in higher IgG compared with long intervals or no vaccination, so that our results are in line with published data ([79,80]).

Both SARS-CoV-2 and HAdV live neutralizing activity capacity of the milk was then analyzed in vitro, resulting in very different behavior of the tested biological samples. First of all, NT50 values for HAdV, a non-enveloped virus, were uniformly zero across all POLI samples, and for all but 2 TS samples, indicating a very low neutralizing activity against the virus. To validate the unexpected results, several human sera from the same mothers were tested, showing high neutralizing titers (1: >256), and confirming the correct execution of the test. In contrast, 72% of POLI colostrum samples, 68.4% of POLI mature milk samples and 43.5% of TS milk samples showed various degrees of neutralizing activity against SARS-CoV-2, an enveloped virus, with NT50 values ranging from, respectively, 1:5.19-1:41.50, 1:2.24-1:20.75, 1:2-1:19. Unfortunately, no results have been published on in vitro neutralizing activity against HAdV of the breast milk, so that we cannot make any comparisons with the literature.

However, similar results were obtained, showing a neutralizing activity of human milk against enveloped viruses and no neutralizing activity against naked viruses such as poliovirus or rotavirus [22,81]. It will be interesting to confirm the different neutralizing activity of human milk against other enveloped and naked viruses, in order to verify whether the composition of the outer layer of the viral particles might have a relevant role in the different behaviors. As known, naked viruses do not possess a lipid envelope that could be disrupted by milk compounds, such as fatty acids. The enzymatic digestion of lipid, through lipases, confers breast milk some of its antiviral properties, this only against enveloped viruses. The antiviral activity seems to be related to the incorporation of the various free fatty acids (medium-chain saturated and long-chain unsaturated fatty acids) into the envelope of these viruses, leading to leakage and their disintegration (REF). Besides, the discrepancy between the NT50 values of SARS-CoV-2 and HAdV could also be due to the experimental conditions explored, in which the effect of other bioactive substances in breast milk, such as the HMOs, or a synergistic action among several substances in breast milk, was not evident, as we will discuss later. Pearson correlation analysis conducted between anti-SARS-CoV-2 IgG and NT_{50} indicated moderate and weak correlation, in the POLI colostrum and POLI mature milk groups, respectively, while a strong positive correlation was found in TS mature milk group.

We hypothesized that these results could be due to three reasons: a) in general, the recent vaccination in the TS group increased the neutralizing activity due to the IgG; b) most likely, the vaccination of the donor mothers conducted in 2021 produced Anti-SARS-CoV-2 IgG levels, highly specific against the B.1 SARS-CoV-2 strain, that were used in our microneutralization test; c) in particular regarding the colostrum, neutralizing activity might be also due to nonspecific compounds, with high defensive properties, abundantly present in the first days of milky whipping. To this regard, a statistically significant difference in NT50 median value against SARS-CoV-2, between POLI colostrum and TS milk groups, was found. These findings are consistent with previous works ([25,26,81]), where human milk was showed to have potent antiviral effect against SARS-CoV-2, even if the mothers have not experienced any SARS-CoV-2 infection ([25,81]). Corroborating this hypothesis, in our study, a group of milk samples, Anti-SARS-CoV-2 IgG negative, showed neutralizing activity against

SARS-CoV-2, indicating the evident effect of other unspecific defensive molecules in milk. Bioactive compounds, such as alfa-lactoalbumin, lactoferrin, HMO, LCPUFAs, cytokine, immunity cells are the complex network that could be the source of neutralizing and inhibitory action. It cannot be forgotten the role of the interindividual differences, for instance due to genes coding for enzymes, involved in the synthesis of oligosaccharides, leading to different HMO profile and to different antimicrobial actions of this specific group of molecules.

In the current study, it was possible to analyze the most abundant, the clinically most relevant and studied, and the most representative entities of each family of fatty acids in the POLI milk group of samples, in order to verify a possible correlation between fatty acid concentration in breast milk and its antiviral effects. Pearson correlation analysis showed a significant correlation between the concentration of Nervonic acid (C24:1) and Lignoceric acid (C24:0) and the neutralizing activity against SARS-CoV-2, with a Pearson coefficient, of 0,36 and 0,30, respectively. The correlation was maintained also when we analyzed separately the colostrum and the mature milk subgroups. Other FAs showed a trend to the positive correlation: Arachidonic acid (C20:4n6), Behenic acid (C22:0) and Stearic acid (C18:0). On the contrary, Palmitoleic acid (16:1n7) showed an intriguing result consisting in a negative correlation, with a Pearson coefficient of -0,34. Even if this result might appear unexpected, it is noteworthy to underline that also other class of molecules, such as HMO, showed a reversed inhibitory effect with the increase of concentration. Further studies are necessary to address the importance of LCPUFAs and the neutralizing activity against enveloped and/or naked viruses, also because many of the properties of fatty acids are still unknown. Additionally, it is important to consider that there is an interplay of the various bioactive molecules, which probably work together to provide the protective antiviral mechanisms, and it is important to consider the temporal changes in milk composition, also during a single day, and some other influencing factors, such as the maternal diet. To this regard, the study of the antiviral properties of human milk may allow the modulation/intensification of its defensive potential, by acting on its specific compounds. The fatty acid profile is in fact influenced by the maternal diet, and through the maternal diet it is possible to modulate its composition.

Finally, the last objective of the research work was to introduce an *in vitro* analysis of the antiviral activity of biosynthesized oligosaccharides, structurally identical to those contained in breast milk. In fact, the prebiotic action of HMOs is known, while the antimicrobial action, and in particular the antiviral one, is still the subject of study and research. Some research has focused on testing individual oligosaccharides, applying them at different time-points, i.e. as pre-treatment of the cells, pre-treatment of the virus, or both, to try to trace the molecular mechanism of viral inhibition, evaluating whether it occurs at the host cells membrane, or at the viral receptor, or both. Other studies have instead been based on molecular kinetic analysis techniques. Within this vast panorama, our choice was to reproduce an *in vitro* test protocol[21], in which a 24-hour cell pretreatment was performed, applying both a powder consisting of several HMOs and a single oligosaccharide (6'SL). In our study, HMO mix showed an increasing antiviral activity (20-50%) when used at the concentration 0.002-0.125 ng/mL, consistently with what was verified by other authors[82] while 6'SL did not show an antiviral effect at the concentrations tested. These results suggest testing the compounds by making variations to the applied protocol, changing, for example, the timing of application of the compounds.

CONCLUSIONS

Although the study described shows some limitations, such as the limited number of samples and the lack of some descriptive characteristics (for example, previous infections, vaccinations, postpartum days), it allowed us to delve deeper into some important issues regarding the antiviral properties of breast milk. Besides, to the best of our knowledge, only other 6 published studies examined the live virus neutralizing capacity of the milk against SARS-CoV-2 and none against HAdV. In fact, most other studies, use pseudovirus assays which are only an approximation of authentic virus neutralization, able only of a single replication cycle, and suffering of high background and variability from the luciferase reporters used. Thus, we were able to confirm some of the already known defensive actions of breast milk against SARS-CoV-2, and to highlight the absence of activity against another respiratory virus, HAdV. Both specific and nonspecific compounds showed a protective effect against SARS-CoV-2. From the correlation analysis, Nervonic and Lignoceric FAs stood out from the rest of the analyzed fatty acids showing a statistically significant positive correlation, while Palmitoleic acid showed a statistically significant negative correlation. Future research, with a larger sample size could investigate the possible antiviral effects of other class of fatty acids that, in our study, showed only a tendency for correlation, also to better explain the presence of neutralizing, but IgG negative, samples.

Future research will be able to verify the neutralizing power of breast milk, HMOs, fatty acids and both in combination, towards other variants of SARS-CoV-2, as well as towards other respiratory viruses. Finally future research could focus on HMO tests, by applying different treatment strategies to the tested cells and tested viruses. Our study also highlights the importance of analyzing the synergistic effects of bioactive compounds in breast milk, as their action as a network has a significant role in antimicrobial and antiviral protection. These efforts and perspectives have as their ultimate goal, the optimization of breastfeeding practices and the improvement of children's health.

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