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Anti-viral Properties of Human Breast Milk: Focus on Glycosaminoglycans composition and Their Role in Pediatric Viral Infections

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1. Abstract

Human milk (HM) is a complex biofluid rich in nutrients and bioactive components essential for infant development and immune protection. Among these components, glycosaminoglycans (GAGs) play a significant role in antiviral defense. GAGs are present in human milk, particularly during the early stages of lactation, and have been shown to inhibit viral infections by acting as decoy receptors. This study focuses on the composition and antiviral activity of GAGs in human milk, specifically investigating their effects on pediatric viral infections such as cytomegalovirus (HCMV) and respiratory syncytial virus (RSV), as well as emerging flaviviruses like Zika virus (ZIKV) and Usutu virus (USUV), which pose potential risks to infant health.

High-performance liquid chromatography (HPLC) and capillary electrophoresis were used to isolate and characterize GAGs from human milk at different stages of lactation. Viral inhibition assays were performed to assess the antiviral activity of these GAGs against HCMV, RSV, ZIKV, and USUV. Additionally, the study explored the binding inhibition of viruses to cells and the impact of human milk components on this process. The results demonstrate that human milk and HM-GAGs exhibit significant antiviral activity, particularly in the early stages of lactation, where concentrations are higher. The composition of HM-GAGs includes heparin (hep), heparan sulfate (HS), chondroitin sulfate (CS), and dermatan sulfate (DS), with varying degrees of sulfation, which influence their antiviral potential.

GAGs were able to inhibit the binding and infection of HCMV, RSV, ZIKV, and USUV to host cells, with the most potent activity observed in colostrum. The inhibition was attributed to the ability of GAGs to act as decoy receptors, preventing viruses from attaching to cell surface receptors.

This study highlights the critical role of GAGs in human milk as antiviral agents, supporting the idea that breastfeeding provides substantial protection against viral infections in infants. The findings suggest that human milk GAGs could be further explored for therapeutic applications, particularly in preventing viral infections during the early stages of infant development.

2. Introduction

The Importance of Breast Milk

Breast milk is not just a source of nutrition for infants; it plays an irreplaceable role in promoting their overall health and development. Its composition is finely tuned to meet the specific nutritional and immunological needs of the newborn, making it far superior to any artificial formula. Breast milk is composed of macronutrients like proteins, fats, and carbohydrates, as well as essential micronutrients such as vitamins and minerals, all of which are critical for infant growth and brain development. For example, the proteins in breast milk, particularly whey and casein, are present in the optimal ratio to be easily digested by the newborn, while also supporting immune function (Victora et al., 2016). Moreover, breast milk is a dynamic biofluid that changes in composition throughout the course of lactation to meet the evolving needs of the infant. In the first few days postpartum, mothers produce colostrum, a yellowish fluid rich in immunoglobulins, especially secretory IgA, which coats the infant's gastrointestinal tract and provides a barrier against infections (Ballard & Morrow, 2013). Colostrum also contains higher concentrations of leukocytes and other immune factors that protect against neonatal infections during this vulnerable period (Brandtzaeg, 2010).

Several studies have highlighted the benefits of breastfeeding in reducing infant mortality and morbidity. Victora et al. (2016) estimated that increasing breastfeeding could prevent over 820,000 child deaths annually worldwide. Breastfed infants are less likely to suffer from acute infections such as respiratory infections, otitis media, and gastroenteritis. For instance, Lamberti et al. (2011) found that breastfed children had a 68% reduction in the risk of pneumonia, a leading cause of mortality in young children. Additionally, breastfeeding has been associated with long-term health benefits, including a reduced risk of obesity, type 2 diabetes, and cardiovascular disease later in life (Horta et al., 2015). Breastfeeding also offers health benefits for mothers. Women who breastfeed have a lower risk of developing breast cancer, ovarian cancer, and postpartum depression. A study by the Collaborative Group on Hormonal Factors in Breast Cancer (2002) found that for every 12 months of breastfeeding, the risk of breast cancer was reduced by 4.3%. Furthermore, breastfeeding helps with postpartum weight loss and reduces the risk of type 2 diabetes, which is especially relevant in societies where obesity and diabetes are prevalent (Stuebe et al., 2010).

Global data from organizations like the World Health Organization (WHO) further support the protective effects of breastfeeding. In regions where exclusive breastfeeding rates are low, higher infant mortality rates are observed, particularly in low- and middle-income countries where access to clean water and proper nutrition is limited (Victora et al., 2016). Therefore, promoting breastfeeding is not just a matter of individual health but also a crucial public health intervention.

In conclusion, breast milk offers unparalleled nutritional and immunological benefits to infants, supporting their growth, protecting against infections, and contributing to long-term health outcomes. The unique combination of bioactive components in breast milk—such as lactoferrin, oligosaccharides, and immunoglobulins—cannot be replicated in formula, underscoring the irreplaceable role of breastfeeding in neonatal care (Lawrence & Pane, 2007).

The Essential Role of Breast Milk in Neonatal Nutrition and Protection Against Infections

Breast milk plays a fundamental role in ensuring both the nutrition and immune protection of newborns. Unlike formula, which lacks many of the bioactive compounds naturally present in breast milk, human milk is perfectly designed to support the infant's development and shield them from a variety of infections during their vulnerable early months. Newborns, particularly preterm infants, are born with immature immune systems that require external support to fight off pathogens. Breast milk provides this support through a variety of immunological factors, bioactive components, and nutritional elements (Victora et al., 2016). One of the key aspects of breast milk is its balanced nutritional composition, which includes proteins, fats, and carbohydrates, alongside a range of essential micronutrients such as vitamins A, D, E, K, calcium, and phosphorus (Ballard & Morrow, 2013). These nutrients are bioavailable and in a form that is easily digested and absorbed by the infant's developing digestive system. For example, breast milk contains both whey and casein proteins in the ideal ratio (60:40) to promote easy digestion and reduce the risk of gastrointestinal disturbances (Lönnerdal, 2003). Moreover, the presence of essential fatty acids, such as docosahexaenoic acid (DHA), is critical for brain and eye development, while lactose, the primary carbohydrate in breast milk, enhances calcium absorption and promotes the growth of beneficial gut bacteria (Innis, 2007).

In addition to nutrition, breast milk provides a protective shield against infections. The colostrum is rich in antibodies, particularly secretory IgA, which coats the infant's mucous membranes and prevents the attachment of pathogens, including viruses and bacteria

(Brandtzaeg, 2010). Studies have shown that colostrum contains significantly higher concentrations of immune cells such as macrophages and neutrophils compared to mature milk, offering immediate protection during the first days of life (Garofalo & Goldman, 1998).

Lactoferrin, a key protein in breast milk, plays a major role in preventing bacterial growth by binding to free iron, a nutrient required by many pathogens for replication (Hanson et al., 2004). Lactoferrin has been shown to inhibit the growth of bacteria such as *Escherichia coli* and *Staphylococcus aureus*, which are common causes of neonatal sepsis (Ochoa & Cleary, 2009). Similarly, breast milk oligosaccharides (HMOs) serve as decoy receptors, preventing harmful pathogens like *Campylobacter* and *Salmonella* from binding to the infant's intestinal cells (Bode, 2012).

Furthermore, research has demonstrated that breastfed infants have a significantly lower risk of respiratory infections. A study conducted in Brazil showed that exclusive breastfeeding reduced the risk of pneumonia by 57% in the first six months of life (Lamberti et al., 2011). Another study found that breastfeeding was associated with a lower incidence of otitis media (middle ear infections), with the risk being 50% lower in exclusively breastfed infants compared to those fed formula (Duncan et al., 2013). The protective effects of breast milk extend beyond infancy, as it has been linked to a reduced incidence of chronic conditions such as asthma, type 1 diabetes, and inflammatory bowel disease (Oddy, 2002).

Globally, the protective role of breast milk has a profound impact on child mortality, particularly in low-resource settings. The World Health Organization (WHO) estimates that exclusive breastfeeding for the first six months could prevent nearly half of all diarrheal diseases and one-third of respiratory infections in young children (WHO, 2018). This makes breast milk an indispensable tool not only for individual health but also as part of public health efforts to reduce child mortality.

The Complex Composition of Breast Milk: Macronutrients and Bioactive Components with Immunological Action

Breast milk is an exceptionally complex fluid that provides a unique combination of essential macronutrients and bioactive components with immunological functions, distinguishing it from any other infant food. This complexity enables it to meet not only the nutritional needs of the infant but also to offer protection against infections and support the development of the immature immune system. The macronutrients in breast milk, such as proteins, fats, and

carbohydrates, are provided in optimal proportions to support healthy growth, as well as the development of the nervous and digestive systems (Victora et al., 2016).

Proteins in breast milk play an essential role both as nutrients and as immune factors. Human milk contains two primary types of proteins: whey and casein, with a ratio of about 60:40 in colostrum and 50:50 in mature milk, facilitating digestion and ensuring adequate protein intake (Ballard & Morrow, 2013). Whey proteins, such as albumin and alpha-lactalbumin, are easily digestible and serve functions beyond simple nutrition. For example, alpha-lactalbumin aids in calcium absorption and lactose synthesis, while albumin helps maintain osmotic balance and transports hydrophobic molecules like fatty acids (Lönnerdal, 2003). In addition to these, breast milk contains immunoglobulins, particularly secretory IgA, which protect the infant's mucosal surfaces, preventing viruses and bacteria from attaching and penetrating the gastrointestinal tract (Brandtzaeg, 2010).

Lipids in breast milk are another crucial component, providing about 50% of the total calories for the infant. Long-chain fatty acids, such as arachidonic acid (ARA) and docosahexaenoic acid (DHA), are critical for brain and retinal development (Innis, 2007). Numerous studies have demonstrated that breastfed infants exhibit better cognitive and visual abilities compared to formula-fed infants, thanks to the presence of these essential fatty acids (Jensen, 1999). Furthermore, lipids in breast milk also serve antimicrobial functions: bile salt-activated lipase releases free fatty acids with antiviral and antibacterial properties, which help destroy pathogens in the intestinal tract (Kabara et al., 1972).

Among the carbohydrates, lactose is the primary sugar in breast milk (fig 1). In addition to providing energy, lactose promotes calcium absorption and plays a key role in maintaining a healthy gut flora by encouraging the growth of beneficial bifidobacteria (Kunz & Rudloff, 1993). Alongside lactose, human milk oligosaccharides (HMOs) have emerged as one of the most studied bioactive components in recent years. HMOs are indigestible by the infant's gut but act as prebiotics, promoting the growth of beneficial bacteria such as bifidobacteria, while also preventing harmful pathogens like *Campylobacter* and *Salmonella* from binding to intestinal cells (Bode, 2012). This dual action of promoting beneficial bacteria and inhibiting pathogens makes HMOs a crucial component of the infant's immune protection (Marcobal et al., 2010).

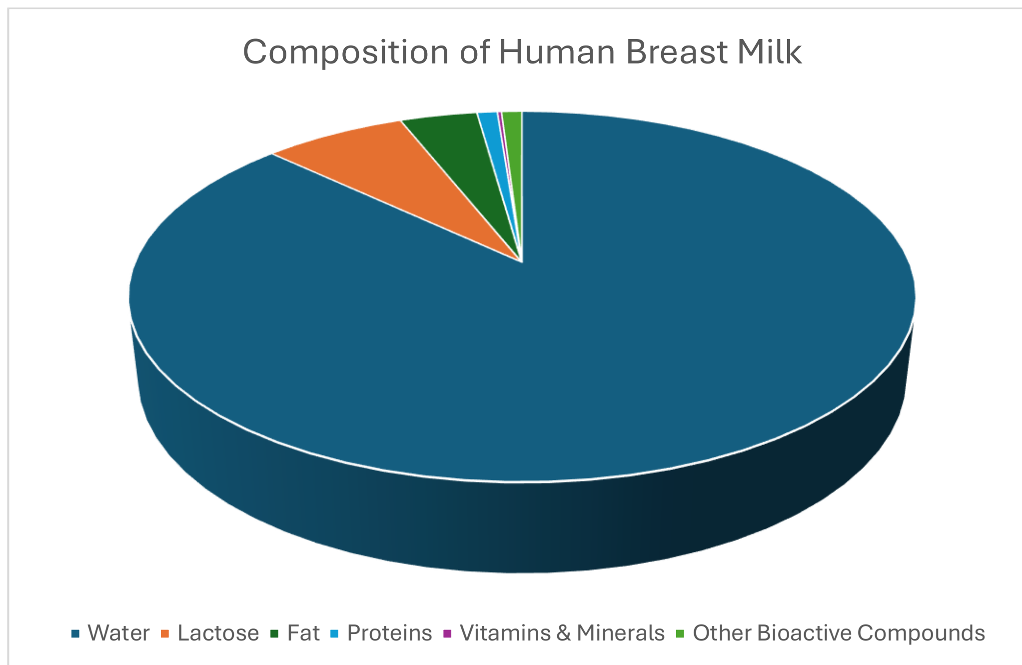

Figure 1: composition of human breast milk

In addition to macronutrients, breast milk is rich in bioactive components with immunological activity, such as cytokines and growth factors. Cytokines, including IL-6 and TNF-α, play a crucial role in modulating the infant's immune response, helping the neonate respond appropriately to pathogens without triggering excessive inflammatory reactions (Garofalo & Goldman, 1998). Growth factors, such as epidermal growth factor (EGF), promote the maturation of the intestines and other mucosal surfaces, accelerating the development of the child's epithelial defenses (Raab & Bell, 2012).

Several studies have highlighted the importance of these immunological components in breast milk in protecting the infant against common infections. For example, a large study by Le Doare et al. (2018) showed that infants exclusively breastfed for the first six months of life had a significantly lower risk of developing respiratory and gastrointestinal infections compared to formula-fed infants. This study underscores how the combined effect of macronutrients and immunological components in breast milk provides integrated protection against a wide range of pathogens.

Virus and Transmission Through Breastfeeding

The transmission of viruses through breastfeeding has been a topic of ongoing research and public health concern, especially with the discovery of various viruses that can be present in human milk. While breastfeeding is universally recognized for its health benefits, certain viruses can potentially be transmitted from mother to infant, raising concerns about the

safety of breastfeeding in specific cases. Viruses such as the human cytomegalovirus (HCMV), human immunodeficiency virus (HIV), respiratory syncytial virus (RSV), Zika virus (ZIKV), and Usutu virus (USUV) have been detected in breast milk, with varying implications for neonatal health (López et al., 2015).

One of the most studied viruses in the context of breastfeeding is HCMV. HCMV is commonly transmitted from mother to infant through breast milk, particularly in preterm infants who are more vulnerable to severe infection. Studies show that up to 40% of breastfed infants born to HCMV-seropositive mothers acquire the virus through breastfeeding (Lazzarotto et al., 2013). However, in most cases, these infections are asymptomatic, as the immunoprotective components in breast milk, such as antibodies and immune cells, often mitigate the severity of the infection. It is generally recommended that mothers continue breastfeeding, even in the presence of HCMV, unless the infant is preterm or immunocompromised (Lazzarotto et al., 2013).

HIV, on the other hand, presents a more complex challenge. Transmission of HIV through breastfeeding has been well-documented, with estimates suggesting that 10-15% of HIV-positive mothers can transmit the virus to their infants through breast milk if no antiretroviral therapy (ART) is provided (WHO, 2010). However, the use of ART in mothers and infants significantly reduces the risk of transmission, and the World Health Organization recommends breastfeeding for HIV-positive mothers in resource-limited settings, as the benefits of breastfeeding outweigh the risks when ART is available (WHO, 2010).

More recently, emerging viruses like ZIKV and USUV have raised concerns about transmission through breastfeeding. ZIKV, which can cause severe neurological defects like microcephaly in newborns, has been detected in breast milk, but current evidence suggests that breastfeeding does not significantly contribute to the vertical transmission of the virus. A case study by Dupont-Rouzeyrol et al. (2016) showed that despite the presence of ZIKV RNA in the breast milk of infected mothers, there was no confirmed transmission of the virus through breastfeeding, leading to the conclusion that breastfeeding should continue even in ZIKV-endemic areas. Similarly, USUV, a lesser-known virus in the flavivirus family, has been detected in breast milk, but there is limited evidence of its transmission through breastfeeding and its potential impact on infant health (López et al., 2015).

The risk of transmission for viruses like RSV through breastfeeding is less understood. RSV primarily spreads through respiratory droplets, but studies have detected viral particles in breast milk. Nonetheless, breastfeeding remains protective against RSV infections due to the

presence of antiviral factors like lactoferrin, which can inhibit viral replication (Hassiotou et al., 2013). In this context, the benefits of breastfeeding in providing immune protection against respiratory infections outweigh the potential risks of transmission.

While certain viruses can be present in breast milk and may pose transmission risks, the overall recommendation remains that breastfeeding should continue in most cases, as the protective effects of breast milk are substantial. Guidelines vary depending on the virus, but in cases like HCMV and ZIKV, the benefits of breastfeeding are often considered to outweigh the risks, particularly when appropriate medical interventions, such as antiretroviral therapy or close monitoring, are in place (Lazzarotto et al., 2013; WHO, 2010).

The detection of viral particles in breast milk has raised numerous questions and controversies regarding the potential for viral transmission from mother to infant. While the presence of viruses like Zika virus (ZIKV), Usutu virus (USUV), human cytomegalovirus (HCMV), and respiratory syncytial virus (RSV) has been confirmed in breast milk, the actual risk of neonatal transmission remains a subject of debate among researchers and healthcare providers. Several factors influence whether the presence of a virus in breast milk leads to infection in the infant, including the viral load, the immunological state of the mother and infant, and the bioactive components present in breast milk that may mitigate transmission.

Zika Virus (ZIKV)

ZIKV, a flavivirus transmitted primarily by mosquitoes, has garnered global attention due to its association with severe congenital abnormalities, such as microcephaly and other neurological defects in newborns. ZIKV RNA has been detected in breast milk, raising concerns about the possibility of vertical transmission through breastfeeding. However, current evidence suggests that the transmission risk is minimal. In a study conducted by Dupont-Rouzeyrol et al. (2016), although ZIKV RNA was found in the breast milk of infected mothers, there were no confirmed cases of transmission to breastfeeding infants. Consequently, the World Health Organization (WHO) advises that breastfeeding should continue in ZIKV-endemic areas, as the benefits of breastfeeding outweigh the theoretical risk of transmission (WHO, 2016). This stance underscores the need for continuous monitoring and research to ensure that public health guidelines adapt to emerging evidence.

Usutu Virus (USUV)

USUV is a lesser-known flavivirus closely related to ZIKV and West Nile Virus (WNV). It has been detected in human breast milk, although there is limited evidence on its

transmission and the clinical consequences for infants (López et al., 2015). The rarity of documented cases and the lack of severe associated outcomes have meant that public health guidelines regarding breastfeeding in USUV-endemic areas remain largely unchanged. The limited data highlights a gap in research on emerging viruses like USUV, particularly concerning breastfeeding safety. As new flaviviruses continue to emerge, public health authorities need to enhance surveillance systems and conduct further studies to assess the risks of breastfeeding transmission and establish evidence-based recommendations.

Human Cytomegalovirus (HCMV)

HCMV is one of the most common viruses transmitted through breastfeeding, particularly among mothers who are HCMV-seropositive. Transmission rates of HCMV through breast milk are as high as 40% in breastfed infants, especially in preterm infants whose immune systems are underdeveloped (Lazzarotto et al., 2013). For most full-term infants, HCMV infections acquired through breastfeeding are asymptomatic due to the protective effects of the immune components present in breast milk. However, preterm infants are at greater risk of developing symptomatic HCMV infection, which can lead to complications such as hearing loss and developmental delays (Lazzarotto et al., 2013). As a result, some healthcare providers recommend pasteurization of breast milk in neonatal intensive care units (NICUs) for preterm infants to reduce the risk of HCMV transmission (Groothuis et al., 2013). Public health efforts must carefully balance the need to protect vulnerable infants while promoting the well-established benefits of breastfeeding.

Respiratory Syncytial Virus (RSV)

RSV is one of the leading causes of lower respiratory tract infections in young children, particularly in the first few months of life. While RSV transmission typically occurs via respiratory droplets, viral particles have also been detected in breast milk (López et al., 2015). The presence of RSV in breast milk does not necessarily mean that breastfeeding transmits the virus; in fact, breastfeeding provides significant protection against RSV infection. Breast milk contains various antiviral components, such as lactoferrin, which inhibits RSV replication and protects the infant's respiratory system (Hassiotou et al., 2013). Public health messaging on breastfeeding and RSV should focus on the protective role of breast milk in preventing severe RSV infections while acknowledging that the virus may be present in milk but with minimal transmission risk.

One of the primary controversies surrounding viral presence in breast milk is the inconsistency in study outcomes regarding transmission risks. For instance, while ZIKV has been detected in breast milk, no concrete evidence supports its transmission to neonates, yet other viruses like HCMV are definitively transmitted through breast milk, particularly in vulnerable populations. This inconsistency has led to differing recommendations regarding breastfeeding in mothers infected with certain viruses.

Furthermore, the role of breast milk's immunological components complicates the discussion. Many studies argue that breast milk's immune factors, such as secretory IgA and lactoferrin, provide robust protection against infection, potentially neutralizing viral particles before they can infect the infant. This immunoprotective effect is one of the main reasons health organizations like the WHO continue to advocate breastfeeding even when viral presence is detected in breast milk (WHO, 2016).

the presence of viral particles in breast milk does not always equate to neonatal transmission. Factors such as the specific virus, viral load, and the infant's immune status all play a role in determining whether transmission will occur. Ongoing research is essential to better understand these dynamics and refine public health recommendations to ensure both the safety of the infant and the promotion of breastfeeding's many benefits (Dupont-Rouzeyrol et al., 2016; Lazzarotto et al., 2013).

Bioactive Components of Breast Milk and Antiviral Action

Breast milk is not only a source of essential nutrients for the infant, but it also contains a range of bioactive components (table 1) that play critical roles in providing protection against viral infections. These bioactive molecules, including immunoglobulins, lactoferrin, oligosaccharides, and glycosaminoglycans (GAGs), contribute to the infant's immune defense by directly neutralizing pathogens or by modulating the infant's immune system to better resist infections. The antiviral properties of breast milk are especially important in the first few months of life, as the infant's immune system is still developing and is less equipped to handle pathogenic threats on its own.

Component	Percentage/Concentration	Function
Water	~87%	Hydrates the infant and serves as the medium for other nutrients.
Lactose	~7%	Primary carbohydrate, provides energy, and promotes healthy gut microbiota.
Fat	~3.8%	Main source of energy, essential for brain development and absorption of fat-soluble vitamins.
Proteins	~1.0%	Includes casein, whey proteins, enzymes, and growth factors that aid in digestion, immune protection, and development.
Vitamins & Minerals	~0.2%	Supports metabolic functions, growth, and overall health. Includes calcium, phosphorus, magnesium, sodium, potassium, iron, zinc, and vitamins A, D, E, K, and B-complex.
Human Milk Oligosaccharides (HMOs)	~1.0%	Enhances gut health, supports immune development, and protects against infections.
Immunoglobulins (sIgA, IgM, IgG)	Trace amounts	Provides immune protection by neutralizing pathogens and preventing infections.
Lactoferrin	Trace amounts	Antiviral, antibacterial properties, binds iron to limit bacterial growth.
Glycosaminoglycans (GAGs)	Trace amounts	Protects against viral infections and supports immune regulation.

Other Bioactive Compounds	Trace amounts	Includes hormones, enzymes, growth factors, and antioxidants that promote overall development and immune function.

Table 1: human milk composition and bioactive components

One of the most potent bioactive components in breast milk are immunoglobulins, primarily secretory IgA (sIgA). Secretory IgA provides critical protection against viral infections by binding to pathogens and preventing them from attaching to and penetrating the infant's mucosal surfaces. This action is especially important in the gastrointestinal and respiratory tracts, which are primary entry points for many viruses. Research has shown that breast milk-derived sIgA provides protection against a variety of viruses, including respiratory syncytial virus (RSV) and enteroviruses, significantly reducing the likelihood of symptomatic infections in breastfed infants (Brandtzaeg, 2010). Unlike systemic immunoglobulins, which enter the bloodstream, sIgA works directly at mucosal surfaces, offering immediate localized protection without triggering inflammatory responses that could harm delicate tissues

Lactoferrin is another key bioactive component with strong antiviral properties. It is an iron-binding glycoprotein and exerts its antiviral effects through several mechanisms. By sequestering iron, lactoferrin deprives many viruses of a necessary nutrient for replication, inhibiting their ability to proliferate. Additionally, lactoferrin can bind directly to viral particles, blocking them from attaching to host cells and initiating infection (Hassiotou & Geddes, 2013). This dual action makes lactoferrin particularly effective against a wide range of viruses, including HIV, herpes simplex virus (HSV), and cytomegalovirus (CMV). Recent studies have highlighted lactoferrin's potential in preventing influenza and coronaviruses, underscoring its significance as a broad-spectrum antiviral agent (Ochoa & Cleary, 2009).

Human milk oligosaccharides (HMOs) are complex carbohydrates unique to human milk, and they play a crucial role in antiviral defense. HMOs function as decoy receptors for pathogens, mimicking the structures that viruses bind to on host cells. By binding to HMOs instead of host cells, viruses like norovirus and influenza are effectively neutralized, reducing their ability to cause infection (Bode, 2012). Additionally, HMOs promote the growth of beneficial gut bacteria, such as bifidobacteria, which play a role in maintaining gut health and further protect the infant from intestinal infections. These prebiotic and

antiviral properties make HMOs a vital component in protecting infants from viral pathogens during the breastfeeding period.

Glycosaminoglycans (GAGs), including heparan sulfate, dermatan sulfate, and chondroitin sulfate, are another group of bioactive compounds in breast milk that contribute to its antiviral properties. GAGs interfere with viral attachment by blocking the receptors on host cells that viruses. For example, heparan sulfate has been shown to block the entry of viruses such as RSV and CMV by inhibiting their attachment to the epithelial cells in the infant's respiratory and digestive tracts (Tollner et al., 2010). Studies also indicate that GAGs can neutralize enveloped viruses, a category that includes herpesviruses and flaviviruses, by disrupting their viral envelopes, which are critical for infectivity. This protective mechanism is particularly valuable in safeguarding infants against viruses that target the respiratory and gastrointestinal systems.

The antiviral components of breast milk do not function in isolation; rather, they work synergistically to enhance the infant's immune defenses. For example, immunoglobulins and lactoferrin both contribute to neutralizing viruses at mucosal surfaces, while HMOs and GAGs prevent viral attachment and facilitate the growth of beneficial microbiota that further support immune health (Hassiotou & Geddes, 2013). This multifaceted approach allows breast milk to offer comprehensive protection, significantly reducing the risk of viral infections during infancy, when the immune system is still developing.

In summary, breast milk's antiviral components, including immunoglobulins, lactoferrin, HMOs, and GAGs, provide infants with a sophisticated immune defense system that not only protects against viral infections but also supports the development of the immune system. This natural antiviral shield, combined with the nutritional benefits of breastfeeding, underscores the critical importance of breast milk in infant health and immunity (Bode, 2012; Brandtzaeg, 2010).

Protective Effects of Breast Milk at Different Stages of Maturation

Human breast milk is a dynamic, biologically active fluid that changes in composition to meet the evolving needs of the infant during the breastfeeding period. The maturation of breast milk can be categorized into three primary stages: colostrum, transitional milk, and mature milk. Each stage of milk development is characterized by distinct changes in the concentration of nutrients and bioactive molecules, which provide tailored immune protection and nutritional support to the infant. Understanding these stages and their unique

properties is crucial for appreciating how breast milk offers a multifaceted defense against infections, supports healthy growth, and strengthens the immune system.

Colostrum: The First Stage of Breast Milk

Colostrum is the earliest form of breast milk produced during the first few days postpartum. It is often referred to as "liquid gold" due to its yellowish color and high concentration of immune-boosting compounds. Colostrum is rich in immunoglobulins, particularly secretory IgA (sIgA), which provides critical protection against pathogens in the infant’s gastrointestinal tract. This early milk is also packed with leukocytes (white blood cells) that help fight infections and promote immune development (Bode, 2012). In addition to its immune components, colostrum contains high levels of lactoferrin, which binds to iron and prevents its use by harmful bacteria, limiting their growth (Lonnerdal, 2003).

Colostrum also has a high concentration of proteins, such as casein and whey proteins, that are easily digestible and provide the infant with essential amino acids needed for growth. The bioactive components in colostrum, including growth factors and cytokines, help protect the infant's gut lining and promote the development of the digestive system. Colostrum is relatively low in fat compared to later stages of breast milk, but this is compensated by the high levels of carbohydrates, particularly lactose, which provides energy and aids in calcium absorption.

Transitional Milk: The Bridge Between Colostrum and Mature Milk

Transitional milk begins to be produced between the second and fifth day postpartum and continues for about two weeks. This stage represents a shift from the nutrient-dense colostrum to more energy-rich mature milk. During this phase, the concentration of proteins begins to decrease, while the fat content increases significantly, providing the infant with the additional energy needed for growth and development. Transitional milk retains many of the immune-protective qualities of colostrum, including high levels of sIgA and lactoferrin, but with gradually increasing volumes to support the growing needs of the infant (Ballard & Morrow, 2013).

One of the notable changes in transitional milk is the increase in lactose concentration. Lactose not only serves as a primary energy source but also plays a role in promoting the growth of beneficial gut bacteria, such as *Bifidobacterium* and *Lactobacillus*, which contribute to the infant’s immune defense. In this stage, the concentrations of oligosaccharides (HMOs), which serve as prebiotics, remain high, continuing to support the

development of a healthy gut microbiota (Newburg, 2009). As the composition of breast milk shifts, transitional milk continues to deliver both nutritional and immunological benefits, adapting to the infant's growing energy needs.

Mature Milk: The Long-Term Nutritional Support

Mature milk develops around two weeks postpartum and remains the primary source of nutrition for the infant during the breastfeeding period. The composition of mature milk stabilizes, although it continues to adapt in response to the infant's needs, such as during illness or growth spurts. Mature milk is lower in protein compared to colostrum and transitional milk, but it contains higher levels of fat, which provides the infant with a rich source of energy for brain development and overall growth. The fat content in mature milk is particularly important for the development of the central nervous system, as it includes essential fatty acids like DHA (docosahexaenoic acid), which is crucial for neural and retinal development (Koletzko et al., 2001).

The carbohydrate content, mainly in the form of lactose and human milk oligosaccharides (HMOs), remains high in mature milk. HMOs play an essential role in the immune system by acting as decoy receptors for pathogens, preventing them from binding to the infant's intestinal cells (Bode, 2012). The HMOs in mature milk continue to promote the growth of beneficial bacteria in the gut, which is critical for immune function and the prevention of infections. Additionally, mature milk contains bioactive compounds such as cytokines, growth factors, and immune cells that support the infant's immune system, reducing the risk of both acute and chronic diseases.

Throughout all stages of lactation, breast milk contains a wide array of bioactive molecules that confer protective effects on the infant. Secretory IgA remains a key immunoglobulin throughout the lactation period, coating the infant's mucosal surfaces and neutralizing pathogens before they can cause infection. Lactoferrin continues to bind iron, preventing bacterial proliferation and providing antimicrobial protection. Lysozyme, an enzyme found in increasing concentrations as milk matures, breaks down bacterial cell walls, contributing to the defense against infections (Lonnerdal, 2003).

Human milk oligosaccharides (HMOs) are also present in all stages of breast milk, with the highest concentrations found in colostrum. These complex carbohydrates not only feed beneficial gut bacteria but also directly inhibit pathogens by acting as decoy receptors. By mimicking the receptors that bacteria and viruses target to enter cells, HMOs prevent

infections, making them a crucial component of the infant’s defense system (Newburg, 2009).

Each stage of breast milk maturation is designed to meet the specific needs of the infant at different stages of development. Colostrum is crucial for providing immediate immune protection and supporting the development of the gut, while transitional and mature milk adapt to provide more energy and continue to offer immune protection through bioactive molecules. As the infant grows, the protective effects of breast milk evolve, ensuring that the infant receives both the nutritional and immunological support necessary for healthy development.

Antiviral Activity Across Different Stages of Lactation: Colostrum, Transitional Milk, and Mature Milk

Breast milk provides powerful antiviral protection throughout lactation, but the concentration and type of bioactive molecules, such as immunoglobulins, lactoferrin, human milk oligosaccharides (HMOs), and glycosaminoglycans (GAGs), change as milk transitions from colostrum to mature milk. These changes reflect the dynamic nature of breast milk, which adapts to the infant's evolving nutritional and immunological needs. Despite fluctuations in the concentration of certain components, the antiviral efficacy of breast milk remains robust across all stages of lactation. In this section, we explore how breast milk's antiviral properties shift through these stages, focusing on the key molecules—particularly GAGs—that contribute to protecting infants from viral infections.

Colostrum: A Potent Early Defense

Colostrum, produced in the first few days after birth, is dense with immune-boosting molecules and serves as the infant's first line of defense against viral infections. Secretory immunoglobulin A (sIgA) is particularly abundant in colostrum, coating the mucosal surfaces of the infant’s digestive and respiratory tracts to prevent viral pathogens from binding to host cells. This early protection is critical in defending against enteric and respiratory viruses, such as rotavirus and respiratory syncytial virus (RSV) (Lonnerdal, 2003; Brandtzaeg, 2010).

Lactoferrin is another crucial bioactive protein found in high concentrations in colostrum, offering broad-spectrum antiviral activity against viruses such as human immunodeficiency virus (HIV), herpes simplex virus (HSV), and cytomegalovirus (HCMV). Lactoferrin acts by sequestering iron—an essential element for viral replication—and by binding directly to

viral particles to block their attachment to host cells (Zhou et al., 2012). The combination of sIgA, lactoferrin, and other proteins makes colostrum an exceptionally potent antiviral agent during the infant's first days of life, when their immune system is underdeveloped and vulnerable to infection.

Glycosaminoglycans (GAGs), including heparan sulfate and chondroitin sulfate, are also highly concentrated in colostrum. These GAGs act as decoy receptors for viruses, preventing them from binding to target cells and initiating infection. This mechanism has been shown to be effective against enveloped viruses like HCMV and RSV (Tollner et al., 2010). Thus, colostrum provides infants with a multi-layered antiviral defense that protects them from a wide range of viral threats.

Transitional Milk: Shifting Defense Mechanisms

As lactation progresses, transitional milk is produced, typically between the second and fifth day postpartum, continuing for about two weeks. Although the volume of milk increases to meet the infant's growing nutritional demands, the concentration of certain immune components, such as sIgA and lactoferrin, begins to decline. However, transitional milk still retains significant antiviral activity, driven by the continued presence of sIgA, lactoferrin, HMOs, and GAGs.

While sIgA levels in transitional milk are lower compared to colostrum, this immunoglobulin remains a key player in antiviral defense, particularly against enteric viruses like rotavirus and enterovirus (Ballard & Morrow, 2013). HMOs also continue to provide critical protection during this stage by acting as decoy receptors that inhibit viral attachment to host cells. This antiviral activity has been observed against viruses such as norovirus and influenza (Bode, 2012).

Although the concentration of GAGs in transitional milk is slightly lower than in colostrum, these molecules still play an essential role in blocking viral entry into cells. Heparan sulfate and dermatan sulfate, two prominent GAGs in breast milk, continue to inhibit viruses such as herpesviruses, Zika virus (ZIKV), and Usutu virus (USUV) by preventing their attachment to epithelial cells (Watterson et al., 2016). This protective mechanism is crucial as the infant's immune system continues to develop.

Mature Milk: Sustained Immunity

Mature milk, produced from two weeks postpartum onward, provides long-term nutritional and immunological support. Although the concentration of bioactive molecules like sIgA

and lactoferrin is lower than in earlier stages, these components remain active in protecting the infant from viral infections. sIgA continues to play a vital role by coating the infant's mucosal surfaces, neutralizing viruses like RSV and rotavirus, while lactoferrin inhibits viral replication by binding to viral particles and limiting iron availability (Zhou et al., 2012). Additionally, lysozyme, an enzyme that enhances antiviral immunity, increases in mature milk, providing further protection (Lonnerdal, 2003).

HMOs remain a critical component of mature milk's antiviral defense, functioning by preventing viral attachment and fostering a healthy gut microbiota. This protection against pathogens such as norovirus and influenza is vital for the infant's gastrointestinal health (Bode, 2012). Although the concentration of GAGs declines in mature milk, heparan sulfate continues to provide antiviral defense by inhibiting the binding of viruses like HCMV and RSV to host cells (Tollner et al., 2010). Even at reduced concentrations, GAGs contribute to the milk's overall antiviral efficacy, working alongside other bioactive components to protect the infant.

Synergistic Antiviral Protection Across Lactation

Breast milk's remarkable ability to offer sustained antiviral protection throughout lactation stems from the combined action of multiple bioactive molecules. While the concentration of specific components may fluctuate, their collective antiviral activity ensures continuous protection against viral infections. The combination of sIgA, lactoferrin, HMOs, and GAGs creates a comprehensive defense system that targets viruses at different stages of the infection process—preventing viral attachment, inhibiting replication, and modulating immune responses.

GAGs, in particular, remain a crucial part of this defense across all stages of lactation. By preventing viruses from entering host cells, GAGs serve as the first line of defense, while other molecules like sIgA and lactoferrin target viruses during later stages of their life cycle. This multi-layered approach is essential for protecting infants during their early months, a critical period for immune development (Tollner et al., 2010; Lonnerdal, 2003).

Breast milk provides sustained antiviral protection through all stages of lactation, adapting to the infant's evolving needs. The presence of sIgA, lactoferrin, HMOs, and GAGs ensures robust defense mechanisms, from colostrum's high concentrations of immune components to mature milk's ongoing support. Together, these bioactive molecules form a synergistic system that offers broad-spectrum protection against viral threats, ensuring the infant's health and development during the critical early months of life.

Glycosaminoglycans and human milk

Glycosaminoglycans (GAGs) are long, unbranched polysaccharides composed of repeating disaccharide units that play essential roles in biological processes such as cell signaling, tissue repair, and immune regulation. These molecules are primarily found in the extracellular matrix of connective tissues and contribute to maintaining tissue structure while facilitating communication between cells. Common types of GAGs include heparan sulfate, chondroitin sulfate, dermatan sulfate, and hyaluronic acid. Notably, GAGs also possess significant bioactive properties, interacting with growth factors and cytokines to modulate inflammation and aid in wound healing. In human breast milk, GAGs are critical in the infant's immune defense, preventing viral attachment to host cells and promoting gut health, thereby contributing to the protective and developmental properties of breast milk (Pomin, 2015).

The Role of Glycosaminoglycans (GAGs) in Human Breast Milk

Glycosaminoglycans (GAGs) play a critical role in various biological processes, including cell signaling, tissue repair, and immune regulation. Found in significant amounts in human breast milk, GAGs contribute to the protective and immunological properties that make breast milk uniquely suited for infant nutrition. These molecules, which include heparan sulfate, dermatan sulfate, and chondroitin sulfate, have been shown to possess potent antiviral, antibacterial, and anti-inflammatory activities, adding to the multifaceted nature of breast milk as not only a nutritional source but also a defense mechanism against infections (Iozzo & Schaefer, 2015; Pomin, 2015).

In the context of breastfeeding, GAGs serve multiple functions that are essential for the health and development of the newborn. One of their primary roles is in protecting the infant from viral infections. GAGs act by preventing viruses from attaching to host cells, a crucial step in the viral replication process. They achieve this by competing with cellular receptors for viral binding, effectively "decoying" viruses away from target cells. This mechanism has been observed in the inhibition of various viruses, including human cytomegalovirus (HCMV) and respiratory syncytial virus (RSV), both of which pose significant health risks to infants (Tollner et al., 2010; Iozzo & Schaefer, 2015).

In addition to their antiviral properties, GAGs also contribute to maintaining the health of the infant's gastrointestinal tract. By promoting the development of a healthy gut microbiota and protecting the intestinal epithelium, GAGs enhance the infant's ability to absorb nutrients while simultaneously preventing the colonization of pathogenic bacteria.

Furthermore, their anti-inflammatory properties help to regulate immune responses in the infant, reducing the likelihood of excessive inflammation that could harm developing tissues (Pomin, 2015; Iozzo & Schaefer, 2015).

The presence of GAGs in breast milk is particularly beneficial for preterm infants, whose immune systems and digestive tracts are underdeveloped. For these infants, GAGs play a vital role in reducing the risk of necrotizing enterocolitis (NEC), a serious gastrointestinal condition, and other complications related to prematurity. By enhancing both immune defense and gut health, GAGs contribute to the overall resilience of the newborn in the critical early months of life (Iozzo & Schaefer, 2015).

The glycosaminoglycans in breast milk offer broad-spectrum protection to infants, functioning as part of a comprehensive system that supports both immune development and resistance to infections. In the following sections, we will delve deeper into the specific types of GAGs found in breast milk, their structures, and the mechanisms through which they exert their protective effects on the newborn (Tollner et al., 2010).

Structure and Composition of Glycosaminoglycans (GAGs): Chondroitin Sulfate, Dermatan Sulfate, Heparan Sulfate, and Heparin

GAGs are linear polysaccharides made up of repeating disaccharide units, typically composed of an amino sugar (such as N-acetylglucosamine or N-acetylgalactosamine) and a uronic acid (such as glucuronic or iduronic acid). These disaccharides are often sulfated, giving GAGs their highly negative charge, which is crucial for their biological functions. The specific sulfation patterns and sugar compositions of GAGs enable interactions with proteins, cytokines, and viruses, contributing to their roles in immune modulation, tissue repair, and infection prevention (Tollner et al., 2010).

Chondroitin Sulfate

Chondroitin sulfate consists of repeating disaccharide units of N-acetylgalactosamine and glucuronic acid, with sulfate groups typically attached at the 4th or 6th position of the N-acetylgalactosamine. These sulfation patterns are critical for its ability to bind to proteins involved in tissue repair and inflammation regulation. Chondroitin sulfate is predominantly found in cartilage, where it helps maintain the structural integrity of tissues by retaining water, contributing to tissue elasticity and compression resistance. In breast milk, chondroitin sulfate may play a role in protecting the infant's epithelial tissues and regulating immune responses (Pomin, 2015).

Dermatan Sulfate

Structurally similar to chondroitin sulfate, dermatan sulfate features N-acetylgalactosamine and iduronic acid in its repeating units. This slight structural variation gives dermatan sulfate different biological properties, particularly in wound healing and coagulation regulation. It is commonly found in skin, blood vessels, and heart valves. Dermatan sulfate also plays a significant role in modulating immune responses and preventing viral attachment to host cells, making it a valuable component of breast milk in protecting the infant's gastrointestinal and respiratory tracts (Iozzo & Schaefer, 2015).

Heparan Sulfate

Heparan sulfate is one of the most complex GAGs in terms of structure, consisting of alternating glucosamine and either glucuronic or iduronic acid. Its sulfation pattern varies, allowing it to interact with a wide range of molecules, including growth factors and viral particles. Heparan sulfate is a key player in cell signaling, particularly in growth factor-mediated pathways such as those involving fibroblast growth factors (FGFs). In breast milk, it has been shown to prevent viral infections by acting as a decoy receptor for viruses like respiratory syncytial virus (RSV) and human cytomegalovirus (HCMV), inhibiting their binding to host cells (Tollner et al., 2010).

Heparin

Heparin, the most sulfated of the GAGs, is structurally similar to heparan sulfate but is more densely packed with sulfate groups. It is well known for its anticoagulant properties and is used medically to prevent blood clotting. Heparin is composed of glucosamine and iduronic acid, with a high degree of sulfation that imparts a strong negative charge. This charge enables heparin to bind to viral envelope proteins, preventing viruses like herpes simplex virus (HSV) and HIV from attaching to host cells. Although present in lower concentrations in breast milk compared to heparan sulfate, heparin contributes to the overall antiviral defense provided by GAGs (Iozzo & Schaefer, 2015).

Structural Diversity and Functional Implications

The structural diversity of GAGs, particularly their sulfation patterns and sugar composition, underpins their varied biological functions. The high density of negative charges due to sulfation allows GAGs to bind to a wide range of positively charged proteins, enzymes, and viral particles, which is essential for their roles in immune regulation, infection prevention, and tissue development. In breast milk, the combined effects of chondroitin sulfate, dermatan

sulfate, heparan sulfate, and heparin provide infants with a multifaceted defense mechanism that supports both immune function and overall health (Iozzo & Schaefer, 2015).

Studies on the Antiviral Effects of GAGs Against ZIKV, USUV, HCMV, and RSV

Glycosaminoglycans (GAGs), such as heparan sulfate, dermatan sulfate, and chondroitin sulfate, have gained attention for their multifaceted role in defending against viral infections. Their ability to interfere with viral attachment, entry, and replication makes them a crucial line of defense, particularly for vulnerable populations like infants, whose immune systems are still developing. In recent years, numerous studies have demonstrated the antiviral properties of GAGs against pathogens like Zika virus (ZIKV), Usutu virus (USUV), human cytomegalovirus (HCMV), and respiratory syncytial virus (RSV). These viruses represent significant public health threats due to their potential for causing severe illness, particularly in newborns and immunocompromised individuals.

Antiviral Mechanisms of GAGs Against Zika Virus (ZIKV)

Zika virus (ZIKV), an arbovirus from the Flaviviridae family, has been linked to serious congenital conditions, including microcephaly, when transmitted from mother to fetus. The primary route of ZIKV transmission is through mosquito bites, but vertical transmission during pregnancy has been documented, which has raised concerns about its presence in breast milk. Studies have highlighted the role of heparan sulfate, a GAG found in breast milk, in inhibiting ZIKV entry into host cells. Heparan sulfate acts as a competitive inhibitor by mimicking the host cell surface receptors that ZIKV typically binds to during the initial phase of infection. This decoy mechanism has been confirmed by Watterson et al. (2016), who showed that heparan sulfate binds to the envelope glycoproteins of ZIKV, preventing the virus from interacting with its primary receptors on human epithelial cells. In vitro studies have demonstrated that the presence of heparan sulfate can reduce ZIKV infectivity by up to 70%, suggesting a significant therapeutic potential. Furthermore, this inhibition is dose-dependent, meaning that higher concentrations of heparan sulfate correlate with a greater reduction in viral attachment and replication. These findings underscore the importance of GAGs in breast milk as a natural defense against ZIKV, particularly in areas where the virus is endemic. Given the serious implications of ZIKV infection in newborns, these results support ongoing research into the protective role of GAGs and their potential use in antiviral therapies or supplements for breastfeeding mothers in ZIKV-endemic regions (Watterson et al., 2016).

Antiviral Role of GAGs Against Usutu Virus (USUV)

Usutu virus (USUV) is another member of the Flavivirus genus, closely related to ZIKV and West Nile Virus (WNV). Although USUV is less studied compared to other flaviviruses, its spread across Europe and Africa has raised concerns about its potential to cause neurological diseases similar to those caused by ZIKV. Preliminary studies indicate that GAGs, such as heparan sulfate and dermatan sulfate, may play a protective role against USUV by preventing viral attachment to host cells.

The structure of USUV is similar to other flaviviruses, which use heparan sulfate as a receptor for initial attachment. Research suggests that GAGs in breast milk can inhibit USUV entry into human cells by binding to viral particles and preventing their interaction with cellular receptors (López et al., 2015). Although specific studies on USUV and GAGs are still limited, the close structural and functional relationship between USUV and other flaviviruses provides a strong basis for hypothesizing similar antiviral effects. Further research is needed to fully elucidate the mechanisms by which GAGs inhibit USUV and to assess the potential clinical applications of these findings in protecting infants in areas where USUV transmission is prevalent.

Glycosaminoglycans and Human Cytomegalovirus (HCMV)

Human cytomegalovirus (HCMV) is one of the most common congenital infections, particularly affecting preterm infants and those with weakened immune systems. Transmission of HCMV through breastfeeding is well-documented, and while the majority of infections in full-term infants are asymptomatic, preterm infants are at risk for serious complications such as sensorineural hearing loss and developmental delays. Heparan sulfate, a GAG abundant in breast milk, has been shown to inhibit HCMV by blocking its interaction with host cell receptors.

HCMV uses heparan sulfate proteoglycans as primary receptors to bind to the epithelial and endothelial cells during the initial stages of infection. Compton et al. (1993) demonstrated that exogenous heparan sulfate competes with these cellular proteoglycans for viral binding, effectively neutralizing the virus before it can enter host cells. This finding was corroborated by subsequent studies showing that GAGs significantly reduce the infectivity of HCMV in both in vitro and in vivo models. By blocking the viral attachment process, GAGs in breast milk may serve as a natural protective mechanism, reducing the risk of vertical transmission of HCMV from mother to infant (Compton et al., 1993).

GAGs' Impact on Respiratory Syncytial Virus (RSV)

Respiratory syncytial virus (RSV) is a leading cause of lower respiratory tract infections in infants, often resulting in bronchiolitis or pneumonia. RSV is highly contagious and is the primary reason for hospitalizations due to respiratory illness in young children. GAGs, particularly heparan sulfate, play a critical role in preventing RSV from binding to epithelial cells in the respiratory tract, thereby reducing the virus's ability to initiate infection. Heparan sulfate and chondroitin sulfate have been shown to interfere with the binding of the RSV attachment protein G, which is essential for viral entry into host cells. Krusat and Streckert (1997) demonstrated that treatment with exogenous heparan sulfate reduced RSV infection rates in human epithelial cell cultures by 70%, a significant finding that highlights the potential for GAGs to mitigate RSV infection in infants. These GAGs not only block viral entry but may also enhance the antiviral immune response by promoting the recruitment of immune cells to the site of infection (Krusat & Streckert, 1997). The presence of such GAGs in breast milk provides an additional layer of defense for breastfed infants, further emphasizing the importance of breastfeeding in protecting against severe RSV infections.

Therapeutic Potential of GAGs in Viral Infections

The antiviral properties of GAGs underscore their potential as both natural defense agents in breast milk and as models for therapeutic development. Given their ability to inhibit viral entry and replication by mimicking host cell receptors, GAGs could be harnessed in antiviral therapies for high-risk populations, such as neonates, preterm infants, and immunocompromised individuals. Their role in neutralizing viral particles before they can establish infection points to a promising future where GAGs may be used as prophylactic agents or treatments for various viral infections.

GAGs have demonstrated significant antiviral by blocking viral attachment to host cells and disrupting the viral life cycle. The presence of these GAGs in breast milk provides infants with a critical defense.

Laboratory Methodologies for the Analysis of Glycosaminoglycans (GAGs) in Human Breast Milk

Due to their complex structure and low concentration in biological fluids, the analysis of GAGs presents unique challenges in terms of extraction, purification, and characterization. This chapter explores the key laboratory methodologies used to isolate and analyze GAGs from human breast milk, with a focus on overcoming the difficulties posed by the complex milk matrix. Techniques such as enzymatic digestion, chromatography, and mass

spectrometry are discussed in detail, providing insights into how researchers can accurately quantify and characterize these important molecules.

Extraction and analysis of GAGs from Human Breast Milk

The extraction of glycosaminoglycans (GAGs) from human breast milk presents several significant challenges due to the complex nature of the milk matrix and the variability in the concentration of GAGs among individual samples. Human breast milk is a highly heterogeneous biological fluid containing various macromolecules, including proteins, fats, carbohydrates, and bioactive compounds. This complexity complicates the isolation of specific molecules like GAGs, necessitating precise and efficient extraction techniques to separate them from other components (Fong et al., 2016; Wu et al., 2018).

Challenges in Extracting GAGs from Breast Milk

1. **Complexity of the Biological Matrix**

   Breast milk contains numerous components, such as proteins, lipids, and carbohydrates, that can interfere with the isolation of GAGs. For example, the high fat content in breast milk forms emulsions, complicating centrifugation and filtration processes. Proteins may aggregate, entrapping GAGs, which hampers their efficient separation. Thus, pre-treatment steps such as fat removal, deproteinization (e.g., using proteolytic enzymes or heat treatments), and clarification are essential to simplify the extraction process (Wu et al., 2018).

2. **Variability in GAG Concentration**

   The concentration of GAGs in breast milk varies significantly depending on factors like the stage of lactation, maternal health, and diet. Colostrum, for instance, generally has higher concentrations of bioactive molecules, including GAGs, compared to mature milk. This variability poses challenges for researchers in developing consistent extraction protocols that work across samples from different individuals and stages of lactation. Adjusting protocols to ensure reproducibility and accuracy is therefore critical (Fong et al., 2016).

3. **Co-extraction of Other Carbohydrates**

   Breast milk contains various carbohydrates, such as lactose and human milk oligosaccharides (HMOs), which may co-extract with GAGs. Given the chemical similarities between HMOs and GAGs, separating these molecules can be difficult

using solubility or charge-based methods. Selective extraction techniques, such as ion-exchange chromatography or filtration, are necessary to minimize contamination by other sugars (Bode, 2012).

4. **Stability of GAGs During Extraction**

   GAGs are prone to degradation during extraction due to their delicate structure. Factors such as temperature, pH, and enzymatic activity can lead to the breakdown of GAGs, altering their molecular weight and bioactive properties. To preserve the native structure of GAGs, mild extraction conditions, such as neutral pH and room temperature, are preferred (Tollner et al., 2010). Protease and glycosidase inhibitors are often used to prevent enzymatic degradation during extraction.

5. **Contamination with Endogenous Enzymes**

   Breast milk contains endogenous enzymes, such as proteases and glycosidases, that can degrade both proteins and polysaccharides. These enzymes can become active during extraction, breaking down GAGs and complicating their isolation. Using enzyme inhibitors to block proteolytic and glycosidic activity is crucial to prevent unwanted degradation and ensure the recovery of intact GAG molecules (Wu et al., 2018).

In summary, the complexity of the milk matrix, variability in GAG concentrations, and potential degradation during extraction present significant challenges. These issues must be addressed to ensure the efficient isolation of GAGs from human breast milk. In the next section, we will discuss specific methodologies developed to overcome these challenges, focusing on the pre-treatment of samples and the use of selective extraction techniques.

Pre-Treatment of the Sample

Before the isolation and analysis of glycosaminoglycans (GAGs) from human breast milk, an essential pre-treatment process is required to remove interfering components and prepare the sample for extraction. Human breast milk contains a complex mixture of proteins, lipids, carbohydrates, and bioactive molecules that can hinder the accurate recovery of GAGs. Effective pre-treatment steps are therefore critical to ensure that GAGs are isolated in their intact form and without contamination. The primary pre-treatment methods include fat removal, deproteinization, and clarification, each of which is necessary to reduce the complexity of the milk matrix and improve the efficiency of downstream extraction processes.

Fat Removal

Human breast milk is rich in lipids, which can account for up to 4% of the milk composition. The presence of fats complicates the isolation of GAGs, as they can form emulsions and interfere with both centrifugation and filtration processes. Therefore, the removal of fat is a crucial first step in sample pre-treatment. One common approach involves centrifugation at low temperatures (typically 4°C), which allows the milk fat to separate from the aqueous layer. This process creates a visible fat layer that can be carefully removed, leaving the aqueous phase containing proteins, carbohydrates, and GAGs (Fong et al., 2016).

Another method involves the use of organic solvents such as chloroform-methanol, which dissolve lipids and separate them from the aqueous phase. This solvent extraction is particularly effective for complete fat removal, but it must be used with caution to avoid disrupting other bioactive molecules in the milk.

Deproteinization

Proteins constitute a significant portion of breast milk and can interfere with the isolation and analysis of GAGs. Deproteinization is therefore a critical step to prevent proteins from co-precipitating with GAGs or binding to them during extraction. Enzymatic digestion using proteolytic enzymes like pronase or trypsin is one of the most effective methods for degrading proteins without damaging GAGs (Wu et al., 2018). These enzymes specifically target and break down protein molecules, leaving GAGs intact for subsequent extraction.

Alternatively, heat treatment can be used to denature proteins. Heating the milk sample to approximately 80°C for a short duration precipitates the proteins, which can then be removed by centrifugation. This method is effective for reducing the protein content in the sample, though care must be taken to ensure that the temperature does not degrade the GAGs (Tollner et al., 2010).

Clarification

After fat removal and deproteinization, the milk sample is typically clarified to remove any remaining particulate matter or aggregates. Filtration through fine-pore membranes (0.22 μm or 0.45 μm) is a common method for clarification. This step ensures that any remaining insoluble materials, such as cell debris or precipitated protein aggregates, are removed, leaving a clear solution containing GAGs and other soluble molecules (Fong et al., 2016).

Alternatively, ultrafiltration can be used to concentrate the GAGs and remove smaller contaminants. This method involves passing the milk sample through membranes with specific molecular weight cut-offs, allowing small molecules to pass through while retaining the larger GAGs. This process not only clarifies the sample but also concentrates the GAGs, improving the efficiency of the extraction process.

Extraction Methodologies

The extraction of glycosaminoglycans (GAGs) from human breast milk involves various techniques designed to isolate these bioactive molecules with minimal contamination. The choice of extraction method depends on factors such as the complexity of the biological matrix, the concentration of GAGs, and the need to preserve their structural integrity. Common methodologies include ethanol precipitation, enzymatic digestion, filtration and centrifugation, and dialysis. Each technique offers specific advantages in isolating GAGs while addressing the challenges of breast milk's complex composition.

Ethanol Precipitation

Ethanol precipitation is one of the most widely used techniques for isolating GAGs from biological fluids. The principle of ethanol precipitation is based on the solubility properties of GAGs in aqueous solutions. GAGs are highly soluble in water but become insoluble when exposed to increasing concentrations of ethanol. By adding ethanol to the milk sample in stepwise increments, GAGs can be precipitated out of solution while other soluble components, such as proteins and carbohydrates, remain in solution.

Procedure:

- Ethanol is typically added to the milk sample to reach a final concentration of 70-80%. This concentration is optimal for precipitating GAGs without co-precipitating large amounts of other molecules.
- After the addition of ethanol, the solution is mixed thoroughly and allowed to incubate at low temperatures (typically 4°C) to enhance the precipitation process.
- The sample is then centrifuged at high speeds to collect the GAG pellet, which is separated from the supernatant. The pellet is washed with cold ethanol to remove residual contaminants.

- The purified GAG pellet is resuspended in an aqueous buffer for further analysis or characterization (Fong et al., 2016).

Ethanol precipitation is favored for its simplicity, scalability, and effectiveness in recovering GAGs in a relatively pure form. However, repeated washing steps are often required to ensure the removal of contaminants, such as proteins and lipids.

Enzymatic Digestion with Specific Enzymes

Enzymatic digestion is a widely used methodology for isolating glycosaminoglycans (GAGs) by selectively degrading other macromolecules present in breast milk. This method is particularly valuable for breaking down complex proteoglycan structures and freeing the GAG chains for further isolation. The enzymes *chondroitinase*, *heparinase*, and *actinase* are commonly employed to target specific GAG subtypes, allowing for precise and effective extraction.

- **Chondroitinase**: This enzyme is used to degrade chondroitin sulfate, a common GAG in breast milk. By cleaving the glycosidic bonds within chondroitin sulfate chains, chondroitinase enables the isolation and analysis of the remaining GAGs in the sample (Kabel et al., 2017). After treatment with chondroitinase, other GAGs like heparan sulfate and dermatan sulfate remain intact, facilitating their further purification.
- **Heparinase**: Heparinase selectively degrades heparan sulfate and heparin, two key GAGs found in breast milk. By breaking down these sulfated polysaccharides, heparinase allows researchers to isolate non-heparan sulfate GAGs or study the degradation products for structural analysis (DeAngelis, 2012).
- **Actinase**: Actinase is another enzyme used to degrade specific protein components associated with GAGs in proteoglycan complexes. This enzyme ensures that GAGs are liberated from their protein moieties, facilitating their isolation in an unbound state for further extraction and analysis (Wu et al., 2018).
- **Pancreatin:** Pancreatin is an enzyme mix that breaks down proteins, fats, and carbohydrates. In the context of GAG isolation, pancreatin helps degrade protein components associated with GAGs in proteoglycan complexes. This process ensures that GAGs are liberated from their protein moieties, facilitating their isolation in an unbound state for further extraction and analysis (Wu et al., 2018).

After enzymatic digestion, the sample is typically processed through filtration or centrifugation to remove the degraded components. The remaining GAGs can then be isolated for subsequent characterization. These enzyme-specific treatments are valuable for targeting and isolating particular GAG types while preserving their structural integrity for downstream applications.

Filtration and Centrifugation

Filtration and centrifugation techniques are widely used for separating GAGs based on their size and molecular weight. Ultrafiltration is one of the most effective methods for isolating GAGs from complex biological matrices like breast milk.

Ultrafiltration

Ultrafiltration involves passing the sample through membranes with defined molecular weight cut-offs, typically ranging from 3,000 to 30,000 Daltons. GAGs, being large polysaccharides, are retained by the membrane, while smaller molecules, such as sugars and salts, pass through.

Procedure:

- The milk sample is first clarified by removing lipids and proteins, as described in the pre-treatment section. The clarified sample is then loaded onto an ultrafiltration device.
- Membranes with specific molecular weight cut-offs are chosen to retain GAGs while allowing smaller contaminants to filter out. The sample is subjected to pressure or centrifugation to pass the liquid through the membrane (Wu et al., 2018).
- The retentate, containing the GAGs, is collected for further purification, while the filtrate, containing smaller contaminants, is discarded or analyzed separately.

This method allows for a high degree of specificity in isolating GAGs based on their molecular size. It is often used in conjunction with other methods, such as chromatography, for further purification.

Dialysis

Dialysis is a technique used to remove small contaminants, such as salts and small organic molecules, from a solution containing GAGs. It is based on the principle of selective diffusion across a semi-permeable membrane, which allows small molecules to pass through

while retaining larger molecules like GAGs. It is useful for desalting samples or removing small solvents that may affect analyses like chromatography or spectrometry. However, it's time-consuming and not ideal for high-throughput use.

Procedure:

- The GAG-containing solution is placed inside a dialysis membrane with a specific molecular weight cut-off (typically 10,000 Daltons). The membrane is then submerged in a buffer or water.
- Over time, small contaminants, such as salts, diffuse out of the dialysis bag, while the GAGs remain inside due to their larger molecular size (Tollner et al., 2010).
- The buffer is changed several times to ensure complete removal of contaminants, leaving a purified GAG solution for further analysis.

Method	Advantages	Disadvantages
Ethanol Precipitation	Simple, scalable, effective for large volumes, relatively low cost	May require multiple washing steps to remove contaminants
Enzymatic Digestion	Highly specific digestion of proteins, preserving GAG integrity	Requires careful control of enzyme activity to avoid degradation
Filtration & Centrifugation	Good for size-based separation; minimizes contamination	Can be time-consuming; requires specialized equipment
Dialysis	Effective at removing small contaminants like salts; preserves larger GAGs	Slow process; not suitable for high-throughput applications

Table 2: GAGs extraction Methods

Purification of GAGs

After the extraction of glycosaminoglycans (GAGs) from human breast milk, purification is necessary to isolate the GAGs from other components that may co-extract, such as proteins, nucleic acids, or other carbohydrates. Several purification techniques are employed to separate GAGs based on their charge, molecular weight, or size. This section discusses key purification methodologies, including ion-exchange chromatography, polyacrylamide gel electrophoresis (PAGE), and size-exclusion chromatography.

Ion-Exchange Chromatography

Ion-exchange chromatography is a widely used method for purifying GAGs based on their charge properties. GAGs are negatively charged polysaccharides due to the presence of sulfate and carboxyl groups. Ion-exchange chromatography takes advantage of this by using a resin that either binds negatively charged molecules (anion exchange) or positively charged molecules (cation exchange). In the case of GAG purification, anion-exchange chromatography is typically used.

Resins in ion-exchange chromatography contain functional groups that interact with the charged molecules in the sample. For GAG purification, a commonly used resin is a strong anion-exchange resin like Q-sepharose, which binds negatively charged GAGs under low-salt conditions. The GAGs can then be selectively eluted by gradually increasing the salt concentration in the elution buffer, which weakens the electrostatic interactions between the GAGs and the resin (DeAngelis, 2012).

In practice, the GAG-containing solution is loaded onto a column packed with the anion-exchange resin under low-salt conditions (e.g., 10-20 mM NaCl). Once the GAGs are bound to the resin, the column is washed to remove unbound contaminants. A gradient of increasing salt concentration (e.g., 0.1-2 M NaCl) is then applied to elute the bound GAGs. The GAGs elute at different salt concentrations depending on their charge density and structure, allowing for the separation of different GAG species (Gandhi & Mancera, 2008).

Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) is used to separate GAGs based on their molecular weight and size. In this technique, the negatively charged GAG molecules migrate through a polyacrylamide gel matrix when an electric field is applied. Smaller molecules move faster through the gel, while larger molecules encounter more resistance and migrate more slowly. PAGE is particularly useful for assessing the purity of GAGs and comparing their molecular weights to known standards.

To assess the molecular weight of the GAGs in the sample, standard GAG markers with known molecular weights are run alongside the sample. This allows researchers to estimate the size of the GAGs based on their migration relative to the standard markers (Volpi et al., 2002). After electrophoresis, the gel is stained using specific dyes, such as alcian blue, which binds to the sulfate groups in GAGs, allowing for their visualization.

Cellulose Acetate Electrophoresis (CAE)

Cellulose acetate electrophoresis (CAE) is a technique used to separate glycosaminoglycans (GAGs) based on their charge and size. In this method, GAGs, which carry a negative charge due to their sulfate groups, migrate across a cellulose acetate membrane when an electric field is applied. The migration speed of the GAGs depends on their charge density and molecular size, with highly charged, smaller molecules moving faster across the membrane. This method is particularly useful for distinguishing between different types of GAGs, such as heparan sulfate and chondroitin sulfate, based on their charge-to-size ratio. After electrophoresis, the cellulose acetate membrane is stained using specific dyes, such as alcian blue, to visualize the GAGs, allowing for their identification and comparison (Volpi et al., 2002).

Size-Exclusion Chromatography (SEC)

Size-exclusion chromatography (SEC), also known as gel filtration chromatography, separates GAGs based on their molecular size. In SEC, the GAG-containing solution is passed through a column packed with porous beads (e.g., Sephadex or agarose gels). Smaller molecules can enter the pores of the beads, slowing their migration, while larger molecules bypass the pores and elute from the column faster. This method is ideal for separating GAGs of different molecular weights (Varki et al., 2009).

Commonly used gels in SEC include Sephadex and Superdex, which are cross-linked dextran gels with different pore sizes. By choosing the appropriate gel with a defined pore size, GAGs of varying molecular weights can be separated and purified. For example, Sephadex G-100 has a fractionation range of 1,000 to 100,000 Daltons, making it suitable for separating large GAG molecules like chondroitin sulfate (Gandhi & Mancera, 2008).

Method	Advantages	Disadvantages
Ion-Exchange Chromatography	Highly effective for separating GAGs based on charge; scalable	Requires careful optimization of salt gradients and resin choice
Polyacrylamide Gel Electrophoresis (PAGE)	Provides clear molecular weight separation; useful for comparing samples	Can be labor-intensive and requires specialized equipment
Size-Exclusion Chromatography (SEC)	Separates GAGs based on molecular size; good for complex mixtures	Resolution can be low for GAGs of similar sizes; requires large sample volumes

Table 3: Gas purification methods

Quantification of GAGs

Once extracted and purified, the quantification of glycosaminoglycans (GAGs) is crucial to understanding their concentration and distribution in human breast milk. Various techniques can be employed to measure GAG levels accurately, including colorimetric methods, UV-Vis spectrophotometry, high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE). Each of these methods offers different advantages based on sensitivity, specificity, and ease of use.

Colorimetric Methods

Toluidine Blue Method

The Toluidine Blue assay is one of the most commonly used colorimetric methods for quantifying GAGs. This method is based on the interaction between the sulfate groups in GAGs and the Toluidine Blue dye, which results in a metachromatic shift in color. When GAGs bind to the Toluidine Blue, the dye shifts from blue to purple, and the intensity of the color change is proportional to the concentration of GAGs in the sample.

- The GAG sample is mixed with a Toluidine Blue solution, and the mixture is incubated for a set period to allow for complete binding of the dye to the sulfate groups.
- The absorbance of the resulting solution is measured at a wavelength specific to the metachromatic shift, typically around 630 nm (Filisetti-Cozzi & Carpita, 1991).
- A standard curve using known concentrations of GAGs is constructed to quantify the amount of GAGs in the sample based on absorbance.

DMB (Dimethylmethylene Blue) Method

The DMB method is specific for sulfated GAGs, such as chondroitin sulfate and dermatan sulfate. The interaction between GAGs and DMB results in a color shift, which can be measured spectrophotometrically. This method is highly specific for GAGs with sulfate groups and is widely used for routine quantification of GAGs in biological samples.

- The sample is mixed with DMB reagent, and the absorbance is measured at 525 nm. The resulting color change is directly proportional to the concentration of sulfated GAGs (Farndale et al., 1986).

- The method is fast and reliable, and a standard curve is used to determine the concentration of GAGs in unknown samples.

UV-Vis Spectrophotometry

UV-Vis spectrophotometry is another method for quantifying GAGs based on their absorption in the ultraviolet (UV) region. GAGs, especially those with strong sulfate or carboxyl groups, exhibit characteristic absorbance peaks in the UV range, which can be used to estimate their concentration.

- GAG samples are dissolved in a suitable buffer, and their absorbance is measured using a UV-Vis spectrophotometer. The absorbance at specific wavelengths (e.g., 230 nm) is indicative of the GAG concentration.
- A standard curve with known GAG concentrations is used to determine the concentration in experimental samples (Volpi, 1993).

UV-Vis spectrophotometry is relatively simple and cost-effective but may lack the specificity of other methods for distinguishing between different types of GAGs.

High-Performance Liquid Chromatography (HPLC)

HPLC is a highly sensitive and precise method used for the quantification of GAGs in complex biological samples such as human breast milk. By using specific detectors, such as UV or refractive index detectors, HPLC can separate and quantify GAGs based on their size and chemical properties.

- The sample is injected into an HPLC system equipped with a specialized column, such as a reverse-phase or ion-exchange column, depending on the type of GAGs being analyzed.
- A gradient of solvents, often aqueous buffers mixed with organic solvents, is used to elute the GAGs from the column.
- Detectors measure the eluted GAGs, and their retention times are compared to standards to quantify the amount of each GAG (Fong et al., 2016).

HPLC is particularly useful for distinguishing between different types of GAGs and is often coupled with mass spectrometry (LC-MS) for further structural analysis.

Capillary Electrophoresis (CE)

Capillary Electrophoresis (CE) is a powerful analytical technique widely used in the separation and analysis of glycosaminoglycans (GAGs). Due to its high resolution, speed, and ability to work with small sample volumes, CE has become increasingly important for GAG analysis in complex biological samples, such as human breast milk. The primary principle of CE involves the movement of charged molecules in an electrolyte-filled capillary under the influence of an electric field. The separation occurs based on the charge-to-size ratio of the molecules.

The separation in CE is driven by two main factors: the electrophoretic mobility of the analytes and the electroosmotic flow (EOF) of the buffer solution inside the capillary. In the context of GAGs, which are highly negatively charged due to their sulfate and carboxyl groups, this method allows for precise differentiation based on the size and charge of the GAG molecules.

1. **Electrophoretic Mobility**:

   Charged molecules move through the capillary when a high-voltage electric field is applied. Larger, more negatively charged GAGs migrate slower, while smaller GAGs or those with fewer negative charges migrate faster. This allows for the separation of various GAG species.

2. **Electroosmotic Flow (EOF)**:

   In CE, the movement of the liquid buffer (EOF) through the capillary also influences the migration of analytes. Since GAGs are negatively charged, the EOF helps drive their movement toward the positively charged electrode at the end of the capillary, enhancing separation efficiency.

Method	Advantages	Disadvantages
Toluidine Blue Method	Simple and effective for measuring sulfate groups in GAGs	May suffer interference from other polysaccharides or proteins
DMB (Dimethylmethylene Blue)	Highly specific for sulfated GAGs; fast and reliable	Limited to sulfated GAGs, not applicable for non-sulfated types
UV-Vis Spectrophotometry	Cost-effective and simple for general quantification	Lacks specificity compared to more advanced techniques

HPLC (High-Performance LC)	Highly precise, capable of separating and quantifying GAGs in complex samples	Expensive equipment and requires trained personnel
Capillary Electrophoresis (CE)	High resolution and sensitivity; effective for small sample volumes	Can be difficult to reproduce without strict control over conditions

Table 4: GAGs quantification methods

Characterization of GAGs

After extraction and purification, the characterization of glycosaminoglycans (GAGs) is crucial for understanding their structural features, molecular composition, and biological functions. Various advanced techniques, such as nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), and immunoassays, are used to precisely determine the sequence, molecular weight, and specific types of GAGs present in samples like human breast milk.

Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy is a powerful tool for characterizing the structure of GAGs at the atomic level. GAGs are composed of repeating disaccharide units, and NMR can provide detailed information about the sequence and arrangement of these sugar residues, as well as their sulfation patterns. By applying NMR, researchers can determine the position of glycosidic linkages, the presence and location of sulfate groups, and the overall three-dimensional structure of GAG chains (Varki et al., 2009).

- In a typical NMR experiment, purified GAG samples are dissolved in a deuterated solvent (e.g., D2O) and placed in a magnetic field.
- When the sample is subjected to radiofrequency pulses, the nuclei (typically 1H, 13C, or 15N) within the GAG molecules resonate at characteristic frequencies based on their local chemical environment.
- These resonances are then measured to generate spectra, which provide insights into the molecular structure of GAGs, including sequence variations and modifications like sulfation or acetylation (Linhardt et al., 2012).

NMR is particularly valuable for characterizing GAGs with complex or heterogeneous structures, as it can resolve detailed structural information that is difficult to obtain using other techniques. However, NMR requires relatively large quantities of purified GAGs and high-resolution instruments, making it a resource-intensive method.

Mass Spectrometry (MS)

Mass spectrometry (MS) is a highly sensitive technique used to determine the molecular weight and composition of GAGs. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry is particularly effective for analyzing large biomolecules like GAGs. By ionizing GAG molecules and measuring their mass-to-charge ratios, MS can precisely determine the molecular weight and identify structural features such as sulfation patterns, acetylation, and other modifications (Zaia, 2005).

- In MALDI-TOF, GAGs are co-crystallized with a matrix material (e.g., a small organic acid) and then subjected to laser pulses, which ionize the GAG molecules without causing significant fragmentation.
- The ionized GAGs are accelerated through a flight tube, and their time of flight is measured. Heavier molecules take longer to reach the detector, while lighter molecules travel faster.
- By measuring the time of flight, the molecular weight of the GAGs can be determined with high accuracy. The technique also allows for the detection of various GAG isoforms and post-translational modifications (Gandhi & Mancera, 2008).

MALDI-TOF is ideal for rapid and accurate analysis of GAGs, especially when determining their molecular weight distribution in complex biological samples like human milk. It can be complemented by other mass spectrometric techniques, such as tandem MS (MS/MS), for detailed structural analysis and sequencing of GAG disaccharide units.

Immunoassay

Immunoassays are widely used for the specific detection and quantification of GAGs in biological samples. These assays rely on antibodies that are highly selective for particular types of GAGs, such as heparan sulfate, chondroitin sulfate, or dermatan sulfate. By targeting these specific epitopes, immunoassays can detect and quantify GAGs even in the presence of other complex molecules.

- An immunoassay typically involves coating a microplate with GAG-specific antibodies, which capture the target GAGs from the sample.
- A secondary detection antibody, often linked to an enzyme such as horseradish peroxidase (HRP), binds to the captured GAGs. A substrate is then added to the

reaction, and the enzyme catalyzes a colorimetric or fluorescent change proportional to the amount of GAGs present in the sample.

- The signal is measured using a plate reader, and the concentration of GAGs is determined by comparison with a standard curve generated using known concentrations of the target GAG (Volpi et al., 2002).

Immunoassays are highly sensitive and specific, making them useful for detecting low concentrations of GAGs in complex mixtures like breast milk. They are also relatively easy to perform and can be adapted to high-throughput formats for screening large numbers of samples.

Method	Advantages	Disadvantages
NMR (Nuclear Magnetic Resonance)	Provides detailed structural information at atomic resolution	Requires large sample volumes and expensive equipment
Mass Spectrometry (MALDI-TOF)	Accurate molecular weight determination and identification of modifications	Can be difficult to interpret for complex mixtures; requires high expertise
Immunoassay	Highly specific detection of GAG types	Dependent on the availability and quality of specific antibodies

Table 5: GAGs characterization methods

Challenges and Considerations

The analysis of glycosaminoglycans (GAGs) in human breast milk presents a series of significant challenges that can complicate both their extraction and characterization. These challenges stem from the inherent variability in biological samples, the complexity of the milk matrix, and the technical limitations of current analytical methodologies. Addressing these issues is crucial for ensuring accurate, reproducible, and meaningful results in the study of GAGs. This section explores the primary challenges in GAG analysis and underscores the importance of standardizing methodologies to achieve comparable outcomes.

Variability Among Individuals

One of the most prominent challenges in the analysis of GAGs is the considerable variability between individuals. GAG concentrations and composition can vary widely depending on several factors, including the stage of lactation, maternal diet, age, genetic factors, and health status. Colostrum, for example, has been shown to have higher concentrations of bioactive molecules, including GAGs, compared to mature milk, but even within similar lactation

stages, substantial differences exist between individuals (Wu et al., 2018). This variability makes it difficult to generalize findings and highlights the need for large sample sizes to capture the full range of GAG diversity.

Additionally, environmental and lifestyle factors, such as maternal stress, illness, or nutrition, can influence GAG levels. These variations complicate efforts to draw firm conclusions about the role and function of GAGs in breast milk. Researchers must account for these individual differences when designing studies and interpreting results, often by employing statistical methods to control for confounding variables. Ensuring sample diversity is critical for studies aimed at understanding the broader implications of GAGs in infant health.

Difficulties in Purification

Another significant challenge in GAG analysis is the complexity of the purification process. Human breast milk is a highly complex biological fluid containing a wide array of proteins, lipids, carbohydrates, and other bioactive molecules. Isolating GAGs from this intricate matrix is technically challenging, as many molecules present in milk can co-purify with GAGs, leading to contamination and interfering with downstream analysis. Furthermore, GAGs themselves are heterogeneous molecules with varying degrees of sulfation, molecular weights, and structures, complicating their separation.

Current purification techniques, such as ion-exchange chromatography and ultrafiltration, are effective but require multiple steps to ensure the removal of contaminants. Each purification step carries the risk of losing GAGs or altering their structure, potentially affecting the accuracy of the final analysis. Furthermore, the delicate balance between preserving the integrity of GAGs and achieving high purity adds another layer of complexity to the purification process (DeAngelis, 2012). Researchers must carefully optimize purification protocols to minimize sample loss and degradation, especially when working with small volumes of breast milk.

Sensitivity of Analytical Techniques

The sensitivity and specificity of analytical techniques are critical in GAG analysis. Due to the low concentration of GAGs in biological samples like breast milk, highly sensitive methods are required to detect and quantify these molecules accurately. Techniques such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy offer high sensitivity and detailed structural information but are expensive, time-consuming, and

require advanced instrumentation. Moreover, they often demand a high degree of expertise to interpret the complex data generated (Linhardt et al., 2012).

Even well-established techniques like colorimetric assays or HPLC face limitations in terms of specificity. Colorimetric methods, for instance, can suffer from interference by other polysaccharides or proteins present in the sample. High-performance liquid chromatography (HPLC) offers high resolution and specificity, but the choice of column and detector can significantly influence the results. Additionally, methods like capillary electrophoresis (CE), although powerful, may struggle with reproducibility if not carefully controlled (Volpi et al., 2002).

The analytical sensitivity of these methods also raises concerns about the detection of subtle differences between GAG isoforms or modifications, such as sulfation patterns, which are crucial for understanding their bioactivity. This underscores the importance of developing more refined and standardized techniques to improve the accuracy and reproducibility of GAG analysis in biological samples.

Standardization of Methodologies

To address the challenges of variability, purification complexity, and analytical sensitivity, there is a pressing need to standardize methodologies for the analysis of GAGs. Currently, many studies use different extraction, purification, and quantification protocols, making it difficult to compare results across studies. The lack of standardized methods can lead to discrepancies in reported GAG concentrations and composition, hindering progress in understanding their role in human health (Gandhi & Mancera, 2008).

Standardization would involve defining best practices for each step of GAG analysis, from sample collection and storage to extraction, purification, and quantification. For example, establishing standardized extraction protocols that minimize sample loss while preserving GAG integrity would enhance the comparability of results between different laboratories. Similarly, agreeing on optimal purification techniques and quality control measures, such as the use of specific markers or reference standards, would ensure that GAGs are consistently isolated in their native form.

Additionally, standardization of analytical techniques is essential for ensuring that GAG quantification is both accurate and reproducible. This could involve adopting specific protocols for HPLC, MS, or NMR analysis, including standardized conditions for sample

preparation, column selection, and detection methods. The use of reference standards and quality control samples would also help to ensure that results are comparable across studies.

3. Methods

This chapter outlines the methodologies employed for the extraction, purification, and analysis of glycosaminoglycans (GAGs) from human breast milk. These techniques are designed to isolate GAGs from the complex matrix of milk and evaluate their antiviral properties against several viral pathogens. Each step is optimized to ensure the integrity and bioactivity of the GAGs, allowing for accurate quantification and detailed characterization.

The aim of this study was to assess the antiviral properties of glycosaminoglycans (GAGs) isolated from human breast milk across different stages of lactation, including colostrum, transitional milk, and mature milk. The primary objectives were to evaluate the concentration of GAGs at each lactational stage, investigate their structural characteristics, and assess their antiviral efficacy against specific viral pathogens such as Zika virus (ZIKV), Usutu virus (USUV), cytomegalovirus (HCMV), and respiratory syncytial virus (RSV).

Participant Enrollment

A cohort of healthy lactating mothers was recruited from the Sant'Anna Hospital (Città della Salute e della Scienza of Turin, Italy). The mothers were stratified based on their lactational stage, contributing samples of colostrum (days 1-5 postpartum), transitional milk (days 6-14 postpartum), and mature milk (beyond day 15 postpartum). Participants included both term and preterm mothers to account for variations in milk composition due to gestational differences.

Sample Collection

Milk samples were collected according to established guidelines for human milk banking. Participants washed their hands and cleaned the breast following Italian Human Milk Bank (HMB) guidelines. Samples were collected in sterile, bisphenol-free polypropylene bottles using a breast pump, ensuring no contamination. After collection, samples were immediately frozen at -20°C and stored until further processing.

Experimental Design

The experimental phase of the study involved multiple steps:

1. **Isolation and Characterization of GAGs**: Fifty milliliters of each milk sample were subjected to a standardized defatting procedure using acetone. The GAGs were then isolated through enzymatic digestion, ethanol precipitation, and dialysis. The isolated GAGs were purified and analyzed using ion-exchange chromatography and capillary

electrophoresis equipped with laser-induced fluorescence to characterize their structural components.

2. **Antiviral Assays**: The antiviral activity of the isolated GAGs was tested using in vitro assays against ZIKV, USUV, HCMV, and RSV. Plaque reduction and focus reduction assays were conducted to determine the effective concentrations (EC50) required to inhibit viral replication. In parallel, cytotoxicity assays were performed to ensure the safety of the GAGs at different concentrations.

3. **Data Collection and Analysis**: The primary outcomes measured were the concentration of GAGs at different lactational stages, their structural integrity after isolation, and their antiviral efficacy. Statistical analyses, including regression models and ANOVA, were performed using GraphPad Prism software to determine the EC50 values and compare the antiviral efficacy across the different milk stages.

Sample Collection and Preparation

A total of 39 healthy lactating mothers participated in the study. The mothers were recruited from Sant'Anna Hospital in Turin, Italy. The selection of participants was based on postpartum stage, allowing the study to include different stages of lactation to capture a diverse set of milk samples: colostrum, transitional, and mature milk.

- Eighteen mothers donated colostrum (milk produced between 1–5 days postpartum). Colostrum is known to be rich in bioactive molecules, including GAGs, which made it a focal point of the study.
- Eleven mothers provided milk samples at different lactation stages. These mothers donated colostrum, transitional milk (6–14 days postpartum), and mature milk (after 15 days postpartum).
- Ten additional mothers each donated 15 ml of mature milk, which was pooled together to create a composite sample for comprehensive analysis.

The collection process adhered to strict hygiene protocols. Before sample collection, all donors thoroughly washed their hands and breasts following the guidelines established by the Italian Human Milk Bank (HMB). Sterile bisphenol-free polypropylene bottles were used to collect the milk, ensuring that no external contaminants interfered with the analysis. The milk samples were collected using an electric breast pump, which was also sterilized before each use to maintain sample purity.

After collection, the milk samples were immediately placed in sterile containers and stored at -20°C. This storage temperature was chosen to preserve the integrity of sensitive bioactive components, including GAGs, until further processing. Samples were kept frozen until the moment of analysis.

- **Pre-Processing and Defatting**:

  Upon thawing, the milk samples were processed to remove fat and cellular debris, ensuring the isolation of the aqueous fraction where GAGs are found. The first step involved centrifugation at 2,000 x g for 10 minutes at room temperature to separate the fat globule layer. The fat was carefully discarded, and the resulting defatted milk was transferred to new, sterile tubes.

- **Centrifugation for Aqueous Fraction Isolation**:

  The defatted milk was further centrifuged at a higher speed (12,000 x g) for 30 minutes. This step allowed for the separation of the aqueous fraction, which contains the dissolved GAGs, from any remaining cellular components and debris. The supernatant, representing the aqueous fraction, was then collected for filtration.

- **Filtration and Final Preparation**:

  To ensure the removal of any residual cells and debris, the aqueous fraction was passed through a sterile syringe equipped with a 0.45 µm pore size filter. This filtration step further purified the sample by eliminating any particulate matter, ensuring that the final solution was free of cellular contaminants and suitable for downstream GAG extraction and analysis.

This detailed pre-treatment process allowed for the consistent isolation of the aqueous phase, where GAGs were concentrated, while minimizing contamination from lipids, proteins, and other components that could interfere with subsequent analyses.

GAG Extraction and purification from Human Milk

The isolation of GAGs from breast milk was achieved through a multi-step process (table 6) involving enzymatic digestion, ethanol precipitation, and dialysis. This methodology ensures the removal of unwanted proteins and contaminants while preserving the structure of GAGs.

To isolate glycosaminoglycans (GAGs) from mature human milk, 50 mL of milk was first subjected to a defatting process using acetone. The milk was centrifuged at 10,000 x g for 15 minutes, and the resulting pellet was dried at 60°C for 24 hours.

Once dry, the pellet was resuspended in 200 mL of distilled water, after which 100 mg of pancreatin (Sigma-Aldrich, code 1071301000) was added. This enzymatic mixture, which contains protease, lipase, and amylase, was stirred at 60°C for 24 hours to break down proteins, lipids, and other interfering components.

Following the enzymatic digestion, the mixture was boiled for 10 minutes to deactivate the enzymes and then centrifuged at 5,000 x g for 20 minutes. To precipitate the GAGs, three volumes of ethanol were added to the supernatant, and the mixture was stored at 4°C for 24 hours. After incubation, the sample was centrifuged again at 10,000 x g for 15 minutes. The resulting pellet was dried at 60°C for 6 hours, then dissolved in 100 mL of 50 mM NaCl solution, and centrifuged at 10,000 x g for 10 minutes to remove any remaining insoluble material.

The supernatant containing the GAGs was loaded onto a column packed with QAE Sephadex A-25, an anion-exchange resin equilibrated with 50 mM NaCl. The GAGs were then eluted using a linear gradient of NaCl, ranging from 50 mM to 2.0 M. The chromatography was performed using a low-pressure liquid chromatography system (BioLogic LP from Bio-Rad) at a flow rate of 1 mL per minute. Fractions positive for uronic acid, indicating the presence of GAGs, were collected.

The GAG-containing fractions were precipitated by adding three volumes of ethanol and stored at 4°C for 24 hours. After this final precipitation step, the samples were centrifuged and dried at 60°C. The dried GAGs were dissolved in distilled water and lyophilized, making them ready for either antiviral testing or further structural analysis.

For antiviral assays, the isolated GAGs were dissolved in phosphate-buffered saline (PBS) and stored at 4°C until use. The purity of the GAG extracts was assessed by measuring protein content using the Folin-Ciocalteu method (Sigma-Aldrich, code MAK365-1KT) and GAG content was quantified using a uronic acid assay.

Thermal treatment of the HM-GAG pool was carried out using a PCR thermocycler (C1000 Thermal Cycler, Bio-Rad). In this process, 100 µl of HM-GAGs were incubated at 63 °C for 30 minutes, followed by rapid cooling at 4 °C. As a control, an equal volume of phosphate-buffered saline (PBS) was subjected to the same conditions. The antiviral activity of the heat-treated GAGs was assessed using a virus inhibition assay, detailed below, and was compared to that of untreated samples.

To further characterize the structural components of the GAGs, the samples were treated with specific enzymes to break down the different types of GAGs. For chondroitin sulfate (CS) and dermatan sulfate (DS) analysis, the GAGs were incubated with chondroitinase ABC or chondroitinase AC in 50 mM Tris-HCl (pH 8.0) for 10 hours at 37°C to release the constituent disaccharides. For heparan sulfate (HS) and heparin (Hep), the samples were treated with a cocktail of heparinases (heparinase I, II, and III) in sodium acetate/calcium acetate buffer (pH 7.0) at 38°C overnight.

The unsaturated disaccharides produced by these enzymatic treatments were then derivatized with 2-aminoacridinone (AMAC), a fluorescent tag. The fluorotagged disaccharides were separated and analyzed using capillary electrophoresis equipped with a laser-induced fluorescence (LIF) detector. This approach not only allowed for the detailed structural analysis of CS, DS, HS, and Hep, but also enabled the detection and quantification of hyaluronic acid (HA). In addition, this analysis provided insights into the charge density and structural characteristics of the sulfated GAGs present in the milk.

Step	Procedure
1. Defatting	50 mL of mature breast milk defatted using acetone. Centrifuged at 10,000 x g for 15 min. Pellet dried at 60°C for 24 hours.
2. Enzymatic Digestion	Pellet resuspended in 200 mL distilled water with 100 mg of pancreatin. Stirred at 60°C for 24 hours to break down proteins and lipids.
3. Enzyme Deactivation	Mixture boiled for 10 min, then centrifuged at 5,000 x g for 20 min.
4. Ethanol Precipitation	Three volumes of ethanol added to supernatant. Stored at 4°C for 24 hours. Centrifuged again at 10,000 x g for 15 min. Pellet dried at 60°C for 6 hours.
5. NaCl Dissolution	Dried pellet dissolved in 100 mL of 50 mM NaCl and centrifuged at 10,000 x g for 10 min.
6. Chromatography	Supernatant loaded onto QAE Sephadex A-25 column. GAGs eluted using NaCl gradient from 50 mM to 2.0 M. Performed with low-pressure chromatography.
7. Final Precipitation	GAG fractions precipitated with ethanol (3 volumes) and stored at 4°C for 24 hours. Centrifuged, dried at 60°C.
8. Lyophilization	Dried GAGs dissolved in distilled water and lyophilized for analysis or antiviral assays.
9. Enzymatic Characterization	GAGs treated with chondroitinase or heparinase enzymes for specific GAG breakdown. Disaccharides derivatized with AMAC and analyzed via capillary electrophoresis with LIF detector.

Table 6: schematic outline of the procedure for isolating glycosaminoglycans (GAGs) from breast milk

A glycosaminoglycan (GAG) library was constructed to evaluate the antiviral properties of different GAGs found in human milk. The library included various types of GAGs, such as chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), and hyaluronic acid (HA). These GAGs were either extracted from human colostrum, transitional, or mature milk samples or purchased as commercially available purified compounds. The library contained both pooled and individual GAGs, allowing for a comprehensive evaluation of their antiviral activities against a variety of viruses. Serial dilutions of each GAG were prepared to assess their dose-dependent effects on viral inhibition. By constructing this GAG library, the study aimed to systematically analyze the contribution of each GAG subtype to the antiviral properties of human milk, with specific attention to how their structure and sulfation patterns influence viral attachment and replication.

Cell Lines and Virus Propagation

Several cell lines were used for the propagation and analysis of different viruses. Vero cells, derived from African green monkey kidney fibroblasts, were cultured in Eagle's minimal

essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C in a 5% CO2 atmosphere. These cells were used for Zika virus (ZIKV) and Usutu virus (USUV) assays.

For HCMV-T and RSV assays, human foreskin fibroblast cells (HFF-1) and human epithelial cells (Hep-2) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Low-passage-number HFF-1 cells were grown in DMEM with 15% FBS, while Hep-2 cells were maintained in DMEM with 10% FBS and 1% Glutamax-I. Both cell lines were incubated at 37°C in a 5% CO2 atmosphere.
Human rotavirus (HRoV) was propagated in MA104 cells, which were also grown in DMEM containing 10% FBS at 37°C. The HRoV Wa strain was activated with porcine pancreatic trypsin before infection.

The ZIKV strains (1947 Uganda MR766 and 2013 French Polynesia HPF2013) were propagated in Vero cells after being generated by transfection of 293T cells. For HCMV, both the Towne strain incorporating GFP and the AD169 laboratory strain were propagated on HFF-1 cells. RSV strain A2 was propagated on Hep-2 cells, and the HRoV Wa strain was propagated on MA104 cells.

For all viruses, titration was performed by plaque assays (ZIKV) or indirect immunostaining (USUV, HCMV, RSV, HRoV) to determine viral concentrations and infectivity, with cells observed using fluorescence microscopy when applicable.

Virus Inhibition Assays

HFF-1, Hep-2, MA104, and Vero cells were seeded in 96-well or 24-well plates depending on the virus being tested. For HCMV, RSV, and HRoV, HFF-1, Hep-2, and MA104 cells were used, while Vero cells were employed for ZIKV and USUV assays. The antiviral activity of human milk-derived GAGs (HM-GAGs) or the aqueous fraction of human milk (colostrum, transitional, or mature milk) was evaluated by incubating serial dilutions of the test compounds with the viruses. Multiplicities of infection (MOIs) were set at 0.02 for HCMV and HRoV, 0.01 for RSV, 0.0005 PFU/cell for ZIKV, and 0.02 FFU/cell for USUV.

The virus-GAG or virus-milk mixtures were incubated for 1 hour at 37°C before being added to the respective cell lines. The mixtures were then incubated for 2 hours in HFF-1 cells (HCMV), 3 hours in Hep-2 cells (RSV), 1 hour in MA104 cells (HRoV), or 2 hours in Vero cells (ZIKV and USUV). After this incubation, the inocula were removed, and the cells were washed twice. HCMV- and RSV-infected cells were overlaid with a 1.2% methylcellulose

medium containing 2% fetal bovine serum (FBS) and incubated for 5 days, while HRoV-infected cells were incubated with fresh medium for 16 hours, and ZIKV- or USUV-infected cells were incubated with methylcellulose or fresh medium for 72 hours and 24 hours, respectively.

For ZIKV, viral plaques were counted after cell fixation and staining with 0.1% crystal violet in 20% ethanol. For HCMV Towne strain, the infected fibroblasts were visualized using fluorescence microscopy, while HCMV AD169, RSV, and USUV-infected cells were fixed with cold methanol–acetone (1:1) and subjected to indirect immunostaining. Viral titers in HRoV-infected cells were also determined by virus-specific immunostaining. The inhibitory dilution (ID50) or effective concentration (EC50) that produced a 50% reduction in viral infection was calculated by comparing treated wells to untreated controls. GraphPad Prism 8.0 software was used to fit dose-response curves and calculate the ID50 and EC50 values.

Viability Assay

Cell viability was assessed using the MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], following the same experimental conditions as the virus inhibition assay. Confluent Vero cells were treated with serial dilutions of colostrum or HM-GAGs. Cell viability was measured using the Cell Titer 96 Proliferation Assay Kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Absorbance was recorded at 490 nm using a Microplate Reader (Model 680, BIORAD). The effect of different dilutions of colostrum or HM-GAGs on cell viability was expressed as a percentage by comparing the absorbance of treated cells to those of control cells incubated with culture medium alone. The 50% cytotoxic concentration (CC50) was determined using GraphPad Prism software.

Immunofluorescence Assay

Subconfluent Vero cells were plated on coverslips in 24-well plates and treated with colostrum at the ID90 concentration, following the virus inhibition assay protocol. Cells were infected with ZIKV or USUV at a multiplicity of infection (MOI) of 3. After 30 hours for ZIKV infection and 24 hours for USUV, the cells were washed twice with PBS and fixed with 4% paraformaldehyde (PAF) for 15 minutes at room temperature. The cells were permeabilized using PBS with 0.1% Triton X-100 for 20 minutes on ice, followed by blocking with 5% bovine serum albumin (BSA) for 30 minutes. Cells were then incubated for 1 hour at room temperature with a primary antibody (Anti-dsRNA or Anti-flavivirus protein E). After three washes with PBS containing 0.05% Tween 20, the cells

were incubated for 1 hour with a rhodamine-conjugated secondary antibody. After three final washes with PBS, the coverslips were mounted and analyzed using a confocal fluorescence microscope (LSM510, Carl Zeiss, Jena, Germany).

Virus Inactivation Assay

For the virus inactivation assays, 150,000 focus-forming units (FFU) of HCMV-T or RSV were incubated with HM-GAGs at concentrations of 80 or 400 µg/mL in a final volume of 200 µL for 1 hour at 37°C. Similarly, approximately 1 x 10^6 FFU of ZIKV or USUV were incubated with 100 µL of colostrum for 2 hours at 37°C. For controls, an equal amount of viral particles was incubated with either culture medium supplemented with PBS (for HCMV-T and RSV) or fresh medium (for ZIKV and USUV).

Following incubation, both treated and untreated viruses were titrated to their respective non-inhibitory dilutions on confluent cells. Residual viral infectivity was measured using fluorescence microscopy for HCMV-T, indirect immunostaining for RSV and USUV, and plaque assays for ZIKV. Viral infectivity was assessed 5 days post-infection for HCMV-T and 3 days post-infection for RSV, while USUV was measured after 24 hours and ZIKV after 72 hours. Statistical analysis was performed using Student's t-test, with significance set at p-values <0.05.

Pre-treatment Assay

For the pre-treatment assay, Vero cells were seeded in 24-well plates at a density of 1.1 x 10^5 cells/well for ZIKV and USUV assays. The following day, confluent cells were pre-treated with serial dilutions of colostrum or HM-GAGs (ranging from 80 to 400 µg/mL for HM-GAGs, or from 1:3 to 1:6561 for colostrum) for 2 hours at 37°C. After pre-treatment, the cells were gently washed twice with warm medium to remove the treatment solution.

Next, the cells were infected with ZIKV, USUV, HCMV-T, or RSV. The viruses were added at a multiplicity of infection (MOI) of 0.0005 PFU/cell for ZIKV, 0.02 FFU/cell for USUV, and an MOI of 3 for both HCMV-T and RSV. The viral inoculum was incubated with the cells for 2 hours at 37°C. After incubation, the cells were washed again to remove unbound virus.

For ZIKV-infected cells, a 1.2% methylcellulose medium was added, and cells were incubated for 72 hours at 37°C. USUV-infected cells were overlaid with fresh medium and incubated for 24 hours. HCMV-T and RSV-infected cells were overlaid with methylcellulose

medium containing 2% fetal bovine serum (FBS) and incubated for 5 and 3 days, respectively.

At the end of the incubation period, ZIKV plaques were visualized and counted after staining with 0.1% crystal violet in 20% ethanol, while USUV, HCMV-T, and RSV-infected cells were assessed by indirect immunostaining or fluorescence microscopy. The percentage of viral inhibition was determined by comparing treated cells to untreated controls, and statistical significance was calculated using Student's t-test, with p-values < 0.05 considered significant.

Binding Assay

Vero, HFF-1, and Hep-2 cells were seeded in 24-well plates at a density of 1.1 x 10^5 cells/well for Vero cells and an appropriate density for HFF-1 and Hep-2 cells. The following day, both the cells and the viruses (ZIKV, USUV, HCMV-T, or RSV, MOI = 3) were cooled to 4°C for 10 minutes. The viruses were then allowed to bind to the cells in the presence of colostrum (ID90) or HM-GAGs (either pooled or individual components) at concentrations of 80, 400, or 2500 µg/mL. After incubation on ice for 1 to 2 hours, the cells were washed with cold medium to remove any unbound viruses.

To release the bound viral particles, the cells were subjected to three rounds of freeze-thaw cycles. The cell lysates were clarified by low-speed centrifugation for 10 minutes. The titers of cell-bound viruses were determined through plaque assay for ZIKV, fluorescence microscopy for HCMV-T, or indirect immunostaining for USUV and RSV. Statistical significance of the results was evaluated using Student's t-test with GraphPad Prism 8.0 software.

Entry Assay

For the entry assay, Vero cells were seeded in 24-well plates at a density of 1.1 x 10^5 cells/well for ZIKV and USUV, while HFF-1 and Hep-2 cells were seeded for HCMV-T and RSV assays, respectively. The following day, both the cells and the viruses (ZIKV, USUV, HCMV-T, or RSV) were cooled to 4°C for 10 minutes to inhibit viral entry, allowing the viruses to bind to the cell surface without internalization.

Viruses were added at a multiplicity of infection (MOI) of 3 for ZIKV, USUV, HCMV-T, and RSV in the presence of serial dilutions of HM-GAGs (ranging from 80 to 2500 µg/mL) or colostrum (ID90). The virus-cell mixtures were incubated on ice for 1 to 2 hours to

facilitate binding. After the incubation period, the cells were washed with cold medium to remove unbound viral particles.

To release the cell-bound virus, cells were subjected to three rounds of freeze-thaw cycles, and the lysates were clarified by low-speed centrifugation. The cell-bound viral titers were then determined through plaque assays for ZIKV, and by indirect immunostaining for USUV, HCMV-T, and RSV. ZIKV plaques were counted after 72 hours of incubation with 1.2% methylcellulose, while fluorescence microscopy was used to visualize HCMV-T-infected cells, and immunostaining was performed for USUV and RSV after 24 hours and 3 days, respectively.

Statistical analysis was conducted using Student's t-test, with significance set at p-values < 0.05.

Post-entry Assay

For the post-entry assay, Vero cells were seeded in 24-well plates for ZIKV and USUV assays, while HFF-1 and Hep-2 cells were used for HCMV-T and RSV assays, respectively. Cells were seeded at a density of 1.1 x 10^5 cells/well (for Vero cells) and allowed to reach confluence the following day.

The viruses (ZIKV, USUV, HCMV-T, or RSV) were added at a multiplicity of infection (MOI) of 3 for ZIKV, USUV, HCMV-T, and RSV, and allowed to infect the cells for 2 hours at 37°C. After the incubation period, unbound virus was removed by washing the cells twice with warm medium. The cells were then treated with serial dilutions of HM-GAGs (ranging from 80 to 2500 µg/mL) or colostrum (at ID90 concentration), which were added to assess their ability to inhibit viral replication after entry into the cells.

For ZIKV-infected cells, a 1.2% methylcellulose medium was added and incubated for 72 hours at 37°C. USUV-infected cells were incubated with fresh medium for 24 hours, while HCMV-T and RSV-infected cells were overlaid with a methylcellulose medium containing 2% fetal bovine serum (FBS) and incubated for 5 and 3 days, respectively. After the incubation, ZIKV plaques were visualized and counted following fixation and staining with 0.1% crystal violet in 20% ethanol. USUV, HCMV-T, and RSV-infected cells were analyzed by indirect immunostaining or fluorescence microscopy to quantify the extent of viral inhibition post-entry.

The effective concentration (EC50) values that produced a 50% reduction in viral replication were calculated by comparing treated cells with untreated controls, and statistical significance was determined using Student's t-test, with p-values < 0.05.

Evaluation of Antiviral Activity of HM-GAGs: Virus Inhibition Assay

The antiviral activity of human milk glycosaminoglycans (HM-GAGs) was evaluated using the virus inhibition assay. Vero cells were seeded, and the following day, cells were pre-treated with serial dilutions of HM-GAGs (ranging from 10 mg/mL to 0.1 mg/mL) for 1 hour at 37°C. Simultaneously, the viruses were pre-treated under the same experimental conditions. Virus-HM-GAG mixtures were incubated for 1 hour at 37°C.The effective concentration (EC50) that produced a 50% reduction in viral infection was calculated by comparing the treated and untreated wells. GraphPad Prism 8.0 software was used to fit a variable slope-sigmoidal dose-response curve and determine the EC50 values.

Data Analysis

All results are presented as the mean values from two to three independent experiments, depending on the specific assay. The ID50 and EC50 values for the inhibition curves were calculated through regression analysis using GraphPad Prism software, version 8.0 (GraphPad Software, San Diego, CA). A variable slope-sigmoidal dose-response curve was fitted to the data to determine these values. Statistical analysis was performed using Student's t-test, analysis of variance (ANOVA), or the F-test, as detailed in the figure legends.

Significance levels were reported based on the specific statistical test employed.

4. Results

Human Milk Antiviral Activity Against ZIKV and USUV

To explore the natural antiviral properties of human milk against ZIKV and USUV, a series of experiments were conducted. Colostrum samples were collected from healthy donor mothers at the Sant'Anna Hospital in Turin, both for term and preterm deliveries. The aqueous fraction of these colostrum samples was tested in vitro against ZIKV (HPF2013 strain) and USUV. Both viruses were treated with serial dilutions of colostrum, before and during infection, to assess antiviral activity.

The results (figure 2a) revealed that all colostrum samples exhibited antiviral properties, although the strength varied between mothers. The ID50 values ranged from 0.0003 to 0.0026 for ZIKV and from 0.0022 to 0.0355 for USUV, showing that colostrum had a significantly stronger effect against ZIKV. Importantly, the ID50 values obtained from freshly processed colostrum were comparable to those from frozen samples, suggesting that storage conditions did not affect the antiviral potency.

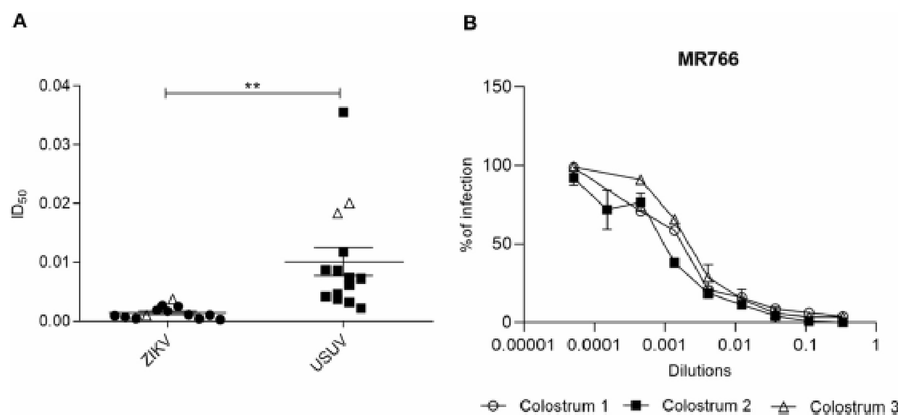

*Figure 2: anti viral activity of defatted colostrum samples. The anti-ZIKV and anti-USUV (a) inhibitory dilution-50 (ID50) values (b). The results are expressed as mean ± SEM of the ID50 values, and statistical significance was determined using the Student's t-test (**p<0.01). The anti-ZIKV activity (b) of three colostrum samples against the African MR766 strain. The dose-response curves illustrate the effect, with the data presented as a percentage of the control. The values are presented as means ± SEM from three independent experiments, each performed in duplicate. White triangle is fresh colostrum (Francese, et al 2020).*

The effect of gestational age on the antiviral activity was evaluated by comparing colostrum samples from term and preterm mothers, but no significant differences in activity against either virus were observed. Furthermore, the antiviral efficacy of colostrum was tested against ZIKV MR766, a strain from the African lineage.

The findings demonstrated that colostrum was also effective in inhibiting MR766 (figure 2b), with ID50 values similar to those observed for the HPF2013 strain ($ID50_{Colostrum1}$ = 0.0012; $ID50_{Colostrum2}$ =0.0009; $ID50_{Colostrum3}$ = 0.0023).

In order to rule out the possibility that the antiviral effect was due to cytotoxicity, viability assays (figure 3) were performed on cells treated with colostrum under the same conditions as the virus inhibition assays. The results confirmed that colostrum was not toxic to the cells, even at the lowest dilution tested (0.33), supporting the conclusion that the antiviral activity of colostrum was not due to cytotoxic effects.

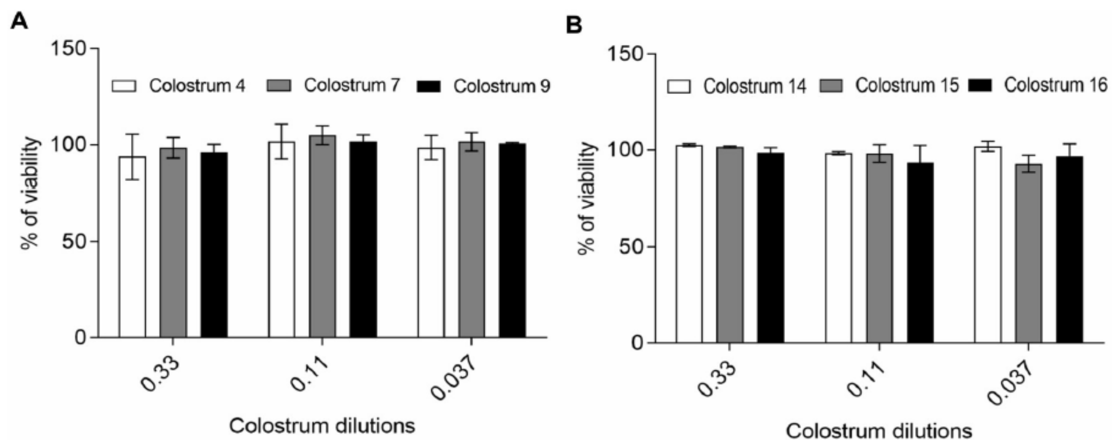

Figure 3: Evaluation of cell viability. Cells were treated under the same conditions as in the ZIKV (a) and USUV (b) inhibition assays. Results are expressed as a percentage of the untreated control. The data represent the means ± SEM of three independent experiments, each conducted in duplicate (Francese, et al 2020).

The antiviral activity of human milk was examined across different stages of lactation. The absence of cytotoxicity was also confirmed for transitional and mature milk samples. Virus inhibition assays (figure 4) showed that all samples had antiviral activity against both ZIKV and USUV, although with varying ID50 values. For ZIKV, the ID50 values ranged from 0.0004 to 0.004 in colostrum, 0.0006 to 0.005 in transitional milk, and 0.0003 to 0.007 in mature milk.

For USUV, the ID50 values ranged from 0.002 to 0.01 in colostrum, 0.001 to 0.007 in transitional milk, and 0.002 to 0.02 in mature milk. While mature milk showed the greatest variation, there was no statistically significant difference in antiviral potency between the different stages of lactation. However, the data confirmed that human milk, particularly colostrum, is more effective against ZIKV than USUV.

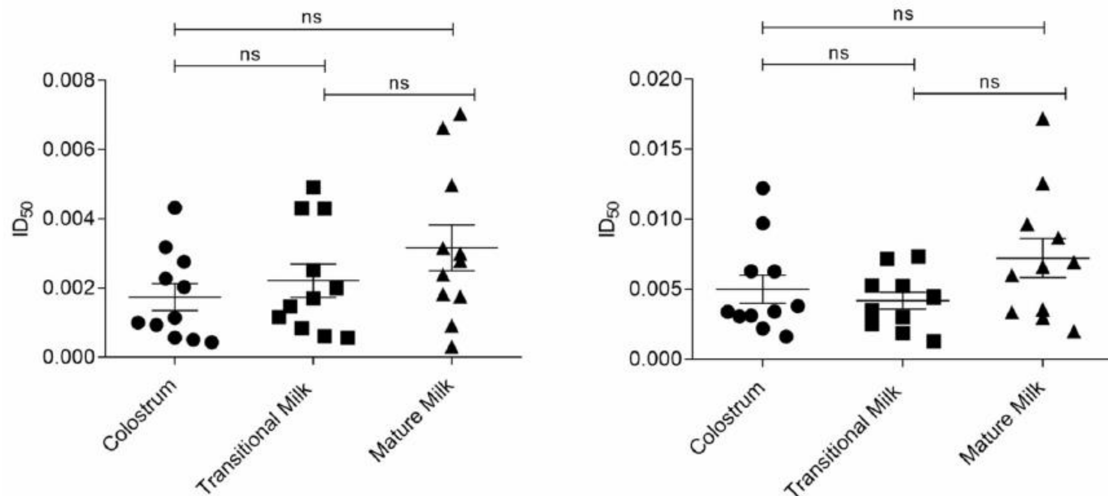

Figure 4: Anti-ZIKV (a) and anti-USUV (b) activity of defatted human milk samples at different stages of lactation. Data are expressed as mean ± SEM and were analyzed using ANOVA followed by the Bonferroni post hoc test (Francese, et al 2020).

The next objective of this study was to identify which stage of the ZIKV and USUV replication cycle is inhibited by human milk (HM). Colostrum was chosen for these experiments due to its overall lower mean ID50 values compared to transitional and mature milk. Initially, we assessed whether the aqueous fraction of colostrum has intrinsic virucidal properties, meaning whether it directly inactivates the viral particles. As shown in figure 5, no significant virucidal activity was detected for either virus.

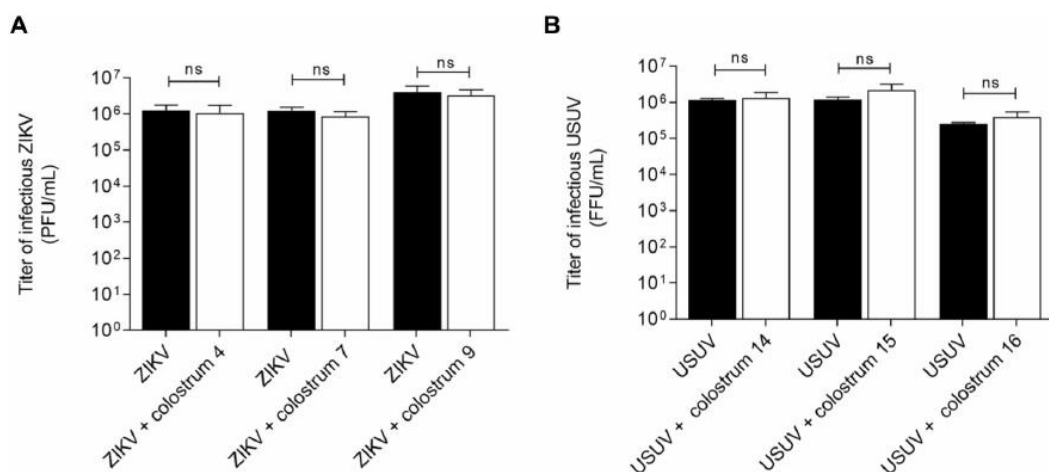

Figure 5: virucidal activity of colostrum aqueous fraction against ZIKV (a) and USUV (b). y-axis shows the infectious titers, expressed as plaque-forming units per ml (PFU/ml) for ZIKV (a) and focus-forming units per ml (FFU/ml) for USUV (b). Error bars represent the standard error of the mean from three independent experiments (Student's t test; ns: not significant) (Francese, et al 2020).

We investigated whether pre-treating cells with serial dilutions of colostrum prior to infection would affect ZIKV and USUV infectivity. The results indicated that this pre-treatment did not impact viral infectivity, suggesting that colostrum does not act directly on the cells to block viral infection (figure 6).

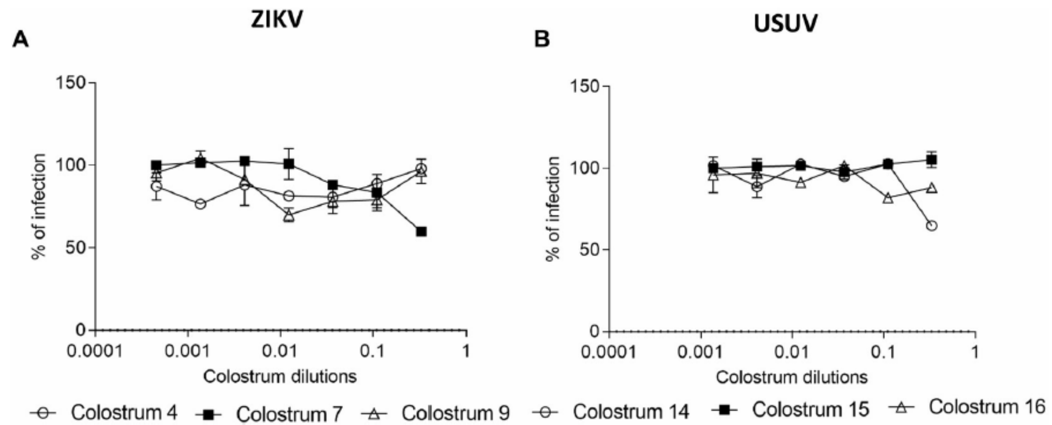

Figure 6: pre-treatment assay with serial dilution of colostrum. The dose-response curves are shown, with the data expressed as a percentage of the control. The values represent the mean ± SEM from three separate experiments, each conducted in duplicate (Francese, et al 2020).

The next focus was on the early stages of viral replication, specifically the binding and entry steps. Colostrum was applied during the virus attachment to cells and during the virus entry process. The data revealed that human colostrum does not interfere with viral entry into cells (figure 7). However, it significantly reduced the binding of both viruses to cells. As demonstrated (figure 8), the viral titer of ZIKV and USUV bound to cells was markedly lower in the presence of colostrum.

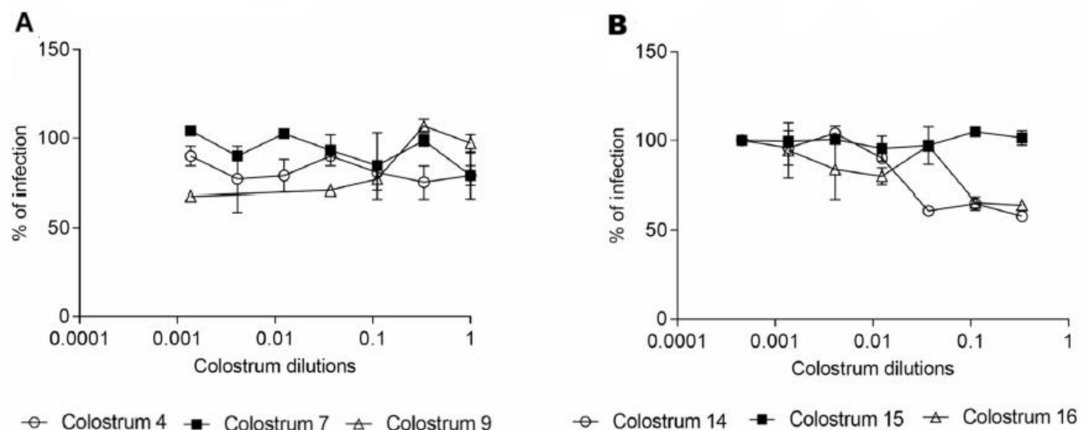

Figure 7: entry assay ZIKV (a) and USUV (b). The dose-response curves are shown, with data expressed as a percentage of the control. Values represent means ± SEM from three independent experiments conducted in duplicate (Francese, et al 2020).

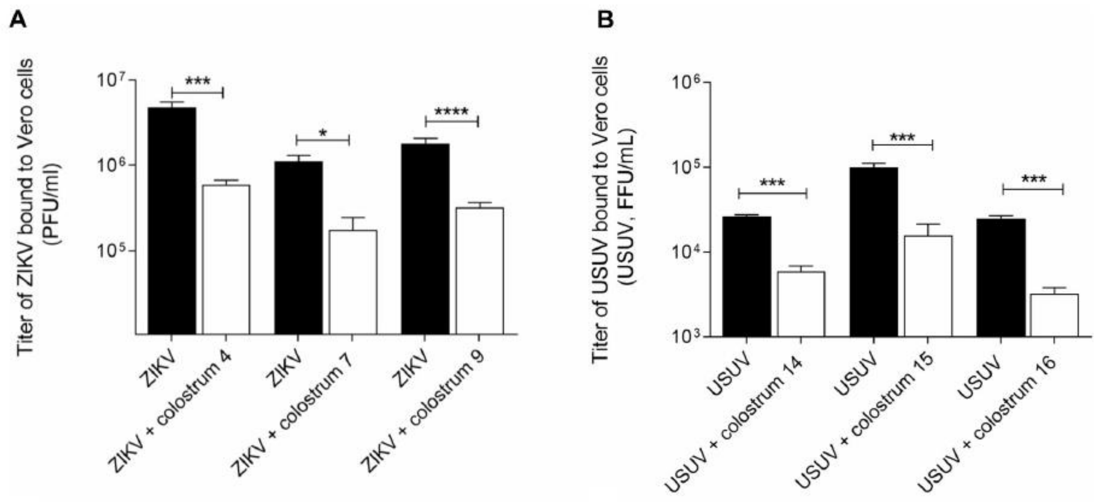

*Figure 8: binding assay ZIKV (a) and USUV (b). y-axis, the infectious titers are represented as plaque-forming units per ml (PFU/ml) for (A) or focus-forming units per ml (FFU/ml) for (B). Error bars indicate the standard error of the mean from three independent experiments (Student's t-test; *p < 0.05; ***p < 0.001; ****p < 0.0001) (Francese, et al 2020).*

Finally, we examined whether colostrum has any effect on the later stages of viral replication. When cells were treated with colostrum after ZIKV or USUV infection for three hours, no decrease in the number of infected cells was observed (figure 9), indicating no additional inhibitory effect at this stage of the viral replication cycle.

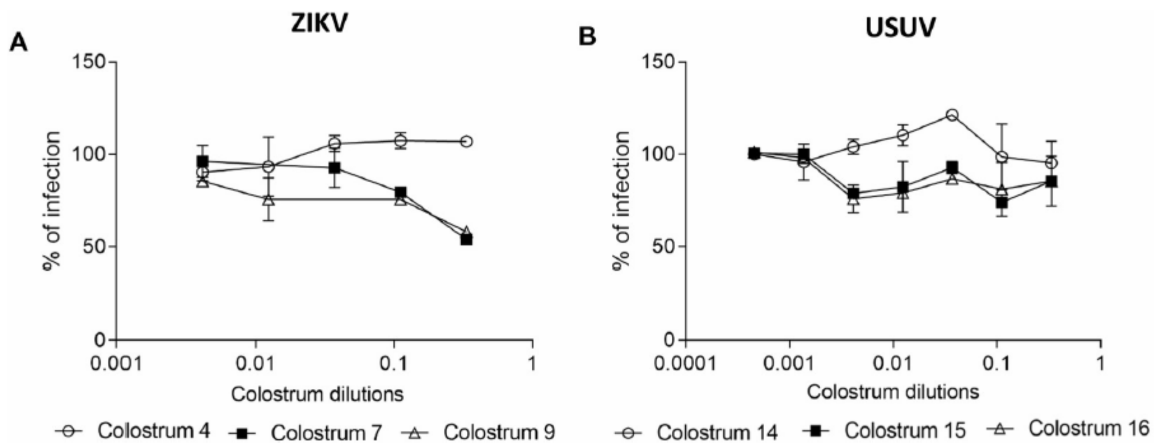

Figure 9: post-entry assay for ZIKV (a) and USUV (b). The dose-response curves are shown, with data expressed as a percentage of the control. Values represent means ± SEM from three independent experiments conducted in duplicate (Francese, et al 2020).

Characterization of Human Milk Glycosaminoglycans

Based on the findings, we aimed to identify additional antiviral components within human milk that contribute to its potent antiviral properties. The focus was placed on isolating and characterizing glycosaminoglycans (GAGs) from mature milk samples, which were

evaluated for their antiviral potency in vitro. Mature milk was chosen for this analysis due to its greater availability compared to colostrum.

A pool of glycosaminoglycans (GAGs) was successfully isolated from mature human milk (HM) samples donated by healthy mothers. The characterization of these isolated HM-GAGs revealed a composition (figure 10) primarily consisting of low-sulfated chondroitin sulfate (CS), which accounted for approximately 55% of the pool, along with about 40% fast-moving heparin (FMHep), and trace amounts of dermatan sulfate (DS), slow-moving heparin (SMHep), and hyaluronic acid (HA). The charge density of the low-sulfated CS was measured at 0.33, which is significantly lower than the typical values for CS from other sources such as bovine milk, which are generally above 0.90.

The FMHep component of the GAG mixture exhibited a charge density of 2.05, which is slightly lower than the reported values for natural heparin samples, typically ranging between 2.40 and 2.90.

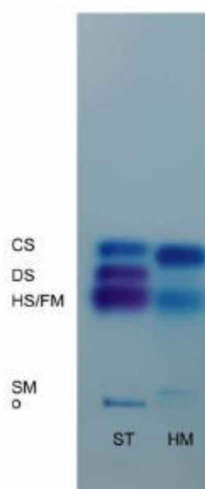

Figure 10: acetate of cellulose electrophoresis of HM-GAGs. ST standard of GAGs, HM human milk, CS chondroitin sulfate, DS dermatan sulfate, HS heparan sulfate, FM fast-moving heparin, SM slow-moving heparin, O origin (Francese, et al 2020).

Antiviral Activity of Glycosaminoglycans in Human Milk Against Pediatric Viral Infections

After the characterization of the GAGs present in human milk, the study focused on determining the antiviral activity of HM-GAGs against Zika virus and Usutu virus, both of

which represent significant health risks to infants. In antiviral assays cells and viruses were exposed to serial dilutions of HM-GAGs both prior to and during infection (figure 11).

The HM-GAGs exhibited activity against both Zika virus (ZIKV) and Usutu virus (USUV) at physiological concentrations typically found in human colostrum. The half-maximal effective concentration (EC50) was determined to be 5.8 mg/ml for ZIKV and 3.3 mg/ml for USUV, with the highest concentration tested (10 mg/ml) able to completely inhibit USUV infection and reduce ZIKV infection by 70%.

Importantly, HM-GAGs samples did not show any cytotoxic effects, suggesting that their antiviral activity was not due to cellular toxicity. These results underscore the significant role that HM-GAGs play, alongside other milk components, in contributing to the intrinsic antiviral properties of human milk against ZIKV and USUV.

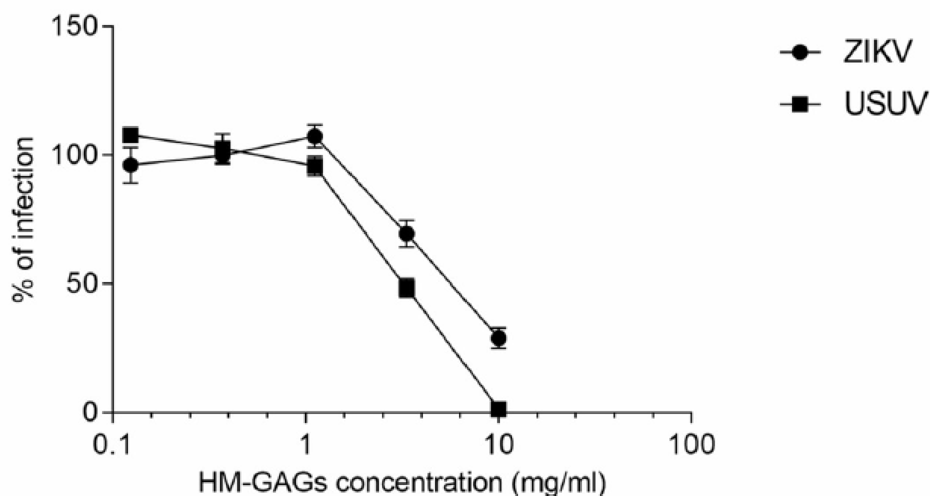

Figure 11: evaluation of anti-ZIKV and anti-USUV activity of HM-GAGs. Cells and viruses were exposed to serial dilutions of HM-GAGs both prior to and during infection. The data are shown as a percentage of the control, and dose-response curves are provided. Results are expressed as means ± SEM from three independent experiments, each conducted in duplicate (Francese, et al 2020).

Subsequently, the investigation into the antiviral activity of HM-GAGs continued, focusing on other pediatric viruses of interest, such as RSV, HCMV, and HRoV. Exposure of two distinct laboratory HCMV strains and RSV to HM-GAGs during the cell-penetration process revealed significant antiviral activity, with effective concentrations (EC50) ranging from 0.37 to 1.89 µg/mL for both viruses (table 7). While no antiviral activity was observed against human rotavirus (HRoV).

To confirm that the observed effects were not due to cytotoxicity, cell viability assays were conducted treating cells with HM-GAGs under the same experimental conditions. HM-

GAGs exhibited no toxicity even at high concentrations (up to 1500 µg/mL). This indicates that the antiviral effects of HM-GAGs are specific to viral inhibition, rather than a result of altered cell health.

Virus	EC50 [µg/ml] (95% CI)	EC90 [µg/ml] (95% CI)	CC50 [µg/ml] (95% CI)	Selectivity Index (SI)
RSV	0.79 (0.67–0.93)	4.23 (2.90–6.32)	>1500	>1899
HCMV-Towne	0.37 (0.25–0.54)	2.98 (1.25–7.09)	>1500	>4054
HCMV-AD169	1.89 (1.21–2.96)	11 (4.16–29.0)	>1500	>794
HRoV	n.a.	n.a.	>1500	n.a.

Table 7: Antiviral activity of HM-GAGs. EC50 effective concentration inhibiting 50% of infection, EC90 effective concentration inhibiting 90% of infection, CC50 half-maximal cytotoxic concentration, CI confidence interval, n.a. not assessable, SI selectivity index

Further investigation into the mechanism of action of HM-GAGs against HCMV and RSV revealed that these GAGs do not possess direct virucidal properties, as demonstrated by virus inactivation assays.

Considering the structural resemblance between HM-GAGs and cellular receptors two potential mechanisms of action were hypotized: either a direct interference with the viral particle itself or a disruption of the virus's ability to bind to host cells. However, a binding assay showed that HM-GAGs inhibit the attachment of viral particles to host cells in a dose-dependent manner. Two concentrations were tested: 80 µg/mL, corresponding to the highest effective concentration (EC99), and 400 µg/mL, representing the lowest physiological concentration found in mature Human Milk. At both concentrations, HM-GAGs significantly reduced the number of virus particles bound to host cells, with over one order of magnitude reduction in viral titers for both HCMV and RSV.

In the virus inactivation assay (figure 12 a and b), HCMV-T or RSV were incubated with HM-GAGs at concentrations of 80 or 400 µg/ml for 1 hour at 37 °C, after which the remaining viral infectivity was measured by titration at the non-inhibitory dilution of HM-GAGs. For the binding assay (figure 12 c and d), HCMV-T or RSV were allowed to attach to cells in the presence of HM-GAGs (80 or 400 µg/ml) for 1 hour on ice.

The titers of cell-bound viruses were then determined through titration on confluent cells. As illustrated (figure 12 a and b), pre-incubating HCMV (Towne strain) and RSV with HM-GAGs did not have any impact on viral infectivity. However, when HM-GAGs were applied to cells at the same time as the viral particles during the binding assay, the viral titers of HCMV and RSV attached to the cells were reduced in a dose-dependent manner (figure 12 c and d). These results suggest that the primary mode of action of HM-GAGs is the prevention of virus attachment, rather than direct inactivation.

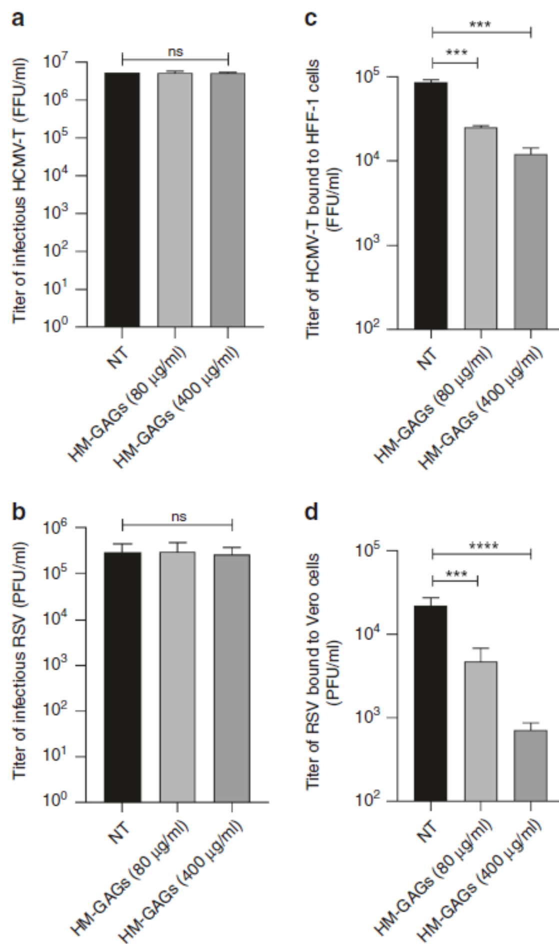

*Figure 12: virus inactivation assay (a, b). Virus binding assay (c, d). On the y-axis, the infectious titers are expressed as focus-forming units per ml (FFU/ml) (a, c) or plaque-forming units per ml (PFU/ml) (b, d). Error bars represent the standard error of the mean from three independent experiments (ANOVA and Bonferroni post hoc test; ***p < 0.001; ****p < 0.0001; ns: not significant) (Francese, et al 2021).*

To delve deeper into the specific contributions of individual GAGs, a library of 12 different GAGs, including CS, DS, FMHep, and others, was screened for antiviral activity against HCMV and RSV.

Table 8 provides a detailed comparison of the anti-HCMV activity of various glycosaminoglycans (GAGs) isolated from human milk. Among the tested GAGs, FMHep

stands out with an exceptionally low EC50 value of 0.09 µg/ml, indicating its potent antiviral activity against HCMV, with a high selectivity index (SI > 55,556), suggesting minimal cytotoxicity. In contrast, chondroitin and DS exhibit relatively higher EC50 values (1729 µg/ml and 60.2 µg/ml, respectively), indicating less potent antiviral activity. Other GAGs like CS1 show moderate activity, while many, including CS2 and CS5, have not shown assessable antiviral effects in this study.

Overall, FMHep is highlighted as the most effective GAG in inhibiting HCMV, showing the greatest promise for therapeutic exploration. Additionally, none of the GAGs tested exhibited cytotoxicity confirming that the observed antiviral effects were not due to toxic effects on the cells.

Type of GAG	EC50 (µg/ml) (95% CI)	EC90 (µg/ml) (95% CI)	CC50 (µg/ml) (95% CI)	SI
CS1	804 (673–961)	>2500	>5000	>6.2
CS2	n.a.	n.a.	>5000	n.a.
CS3	n.a.	n.a.	>5000	n.a.
CS4	n.a.	n.a.	>5000	n.a.
Chondroitin	1729 (1272–2350)	>2500	4824	>2.8
HA 1.500 kDa	n.a.	n.a.	>5000	n.a.
HA 1.000 kDa	n.a.	n.a.	>5000	n.a.
HA 200 kDa	n.a.	n.a.	>5000	n.a.
HA 4.6 kDa	n.a.	n.a.	>5000	n.a.
CS5	n.a.	n.a.	>5000	n.a.
FMHep	0.09 (0.07–0.14)	0.53 (0.26–1.07)	>5000	>55,556
DS	60.2 (33.4–109)	1941 (448–8412)	>5000	>83.1

Table 8: anti-HMCV activity of different types of GAGs
EC50: Effective concentration inhibiting 50% of infection. EC90: Effective concentration inhibiting 90% of infection. CC50: Half-maximal cytotoxic concentration. SI: Selectivity Index. n.a.: Not assessable

Table 9 presents data on the antiviral activity of different glycosaminoglycans (GAGs) against the respiratory syncytial virus (RSV). FMHep (fast-moving heparin) and dermatan sulfate (DS) demonstrated significant inhibitory effects, with FMHep showing an EC50 value of 0.99 µg/ml and DS having an EC50 of 22.75 µg/ml, making them the most effective GAGs tested. Chondroitin and CS1 also exhibited some antiviral activity, although at much higher concentrations.

Importantly, none of the tested GAGs showed cytotoxicity, as their CC50 values exceeded 5000 µg/ml, indicating that their antiviral effects were not due to cellular toxicity. Overall, FMHep and DS stand out as the most potent inhibitors of RSV in the GAG library.

Type of GAG	EC50 (µg/ml) (95% CI)	EC90 (µg/ml) (95% CI)	CC50 (µg/ml) (95% CI)	SI
CS1	686.9 (510.1–1029)	>2500	>5000	>7.28
CS2	n.a.	n.a.	>5000	n.a.
CS3	n.a.	n.a.	>5000	n.a.
CS4	n.a.	n.a.	>5000	n.a.
Chondroitin	104.3 (57.89–180.7)	1470 (475.5–10,009)	>5000	>47.94
HA 1.500 kDa	n.a.	n.a.	>5000	n.a.
HA 1.000 kDa	n.a.	n.a.	>5000	n.a.
HA 200 kDa	n.a.	n.a.	>5000	n.a.
HA 4.6 kDa	n.a.	n.a.	>5000	n.a.
CS5	1582 (956.4–4455)	>2500	>5000	>3.16
FMHep	0.99 (0.7–1.4)	41.75 (16.87–103.3)	>5000	>5051
DS	22.75 (16.26–31.54)	316.7 (144–781)	>5000	>219.78

Table 9: anti-RSV activity of different types of GAGs. EC50: Effective concentration inhibiting 50% of infection. EC90: Effective concentration inhibiting 90% of infection. CC50: Half-maximal cytotoxic concentration. SI: Selectivity Index. n.a.: Not assessable

We further explored the capacity of HM-GAGs to inhibit viral binding to host cells. As shown in figure 13 a, CS1, chondroitin, and DS significantly impaired HCMV binding when tested at a concentration of 2500 µg/ml. FMHep demonstrated the strongest inhibitory effect, with just 100 µg/ml reducing the titer of HCMV bound to cells.

Similarly, CS1, chondroitin, and DS (at 2500 µg/ml) significantly affected RSV binding to Hep-2 cells, though CS5 did not show a notable effect on RSV. Consistent with the HCMV results, FMHep was also the most effective GAG in inhibiting RSV binding, with 100 µg/ml leading to a reduction in titer by more than one order of magnitude, comparable to the total HM-GAG preparation (figure 13 b).

Collectively, these findings suggest that individual HM-GAGs may work synergistically to disrupt HCMV and RSV binding to host cells, with FMHep exerting the strongest effect.

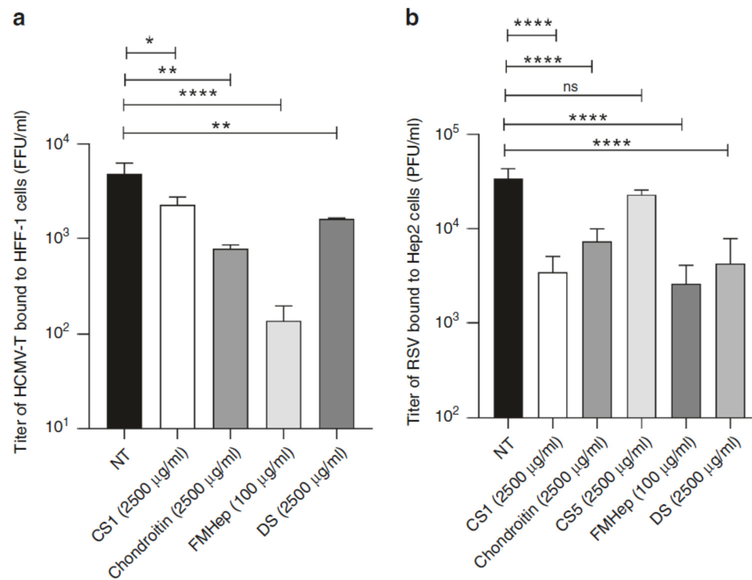

*Figure 13: Binding assay. The y-axis displays the infectious titers as focus-forming units per milliliter (FFU/ml) for graph (a) and plaque-forming units per milliliter (PFU/ml) for graph (b). The error bars represent the standard error of the mean (SEM) from three independent experiments. Statistical significance was determined using ANOVA followed by Bonferroni's post hoc test, with significance levels indicated as follows: *p < 0.05, **p < 0.01, ****p < 0.0001, and "ns" for not significant (Francese, et al 2021).*

Heat Treatment and Stability of GAGs' Antiviral Activity

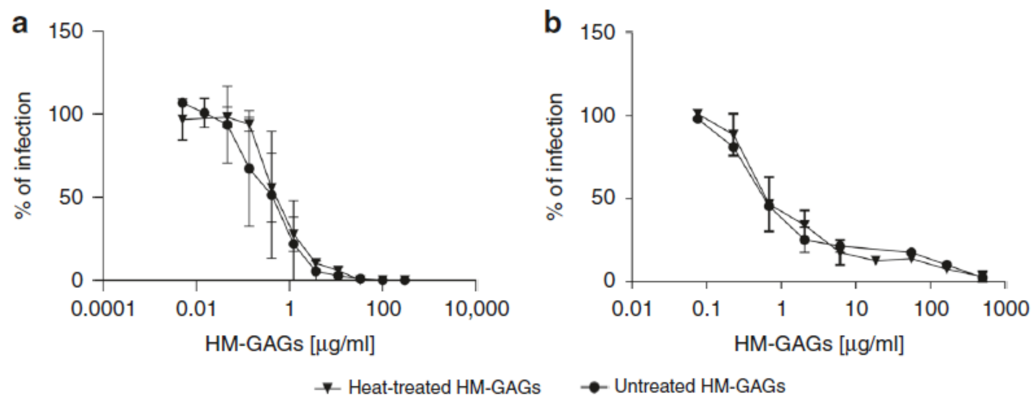

Figure 14: Anti-HCMV (a) and anti-RSV (b) activity of heat-treated HM-GAGs. A virus inhibition assay was then performed by treating HCMV-T (a) or RSV (b) with either heat-treated or untreated HM-GAGs prior to and during the cell penetration phase. The dose-response curves are shown, with data presented as a percentage relative to the control (untreated virus). The values represent the means ± SEM of three independent experiments, each performed in triplicate. The EC50 values for the heat-treated and untreated HM-GAGs were calculated as 1.03 and 0.79 µg/ml for RSV, and 0.58 and 0.37 µg/ml for HCMV, respectively. A comparison of the data using an F-test revealed no significant differences between the two treatments (Francese, et al 2021).

An additional focus was on the stability of GAGs' antiviral properties after heat treatment. Since donor milk is often subjected to pasteurization in human milk banks, it was crucial to evaluate whether pasteurization (HoP) would affect the antiviral efficacy of GAGs. The

GAGs were subjected to Holder pasteurization conditions (63°C for 30 minutes), and subsequent antiviral assays were conducted.

The EC50 values for heat-treated and untreated HM-GAGs were calculated, with values of 1.03 and 0.79 μg/ml for RSV, and 0.58 and 0.37 μg/ml for HCMV, respectively. Results showed (figure 15) that the antiviral activity of GAGs was maintained after pasteurization, suggesting that pasteurized donor milk retains its protective effects against viral infections such as HCMV and RSV.

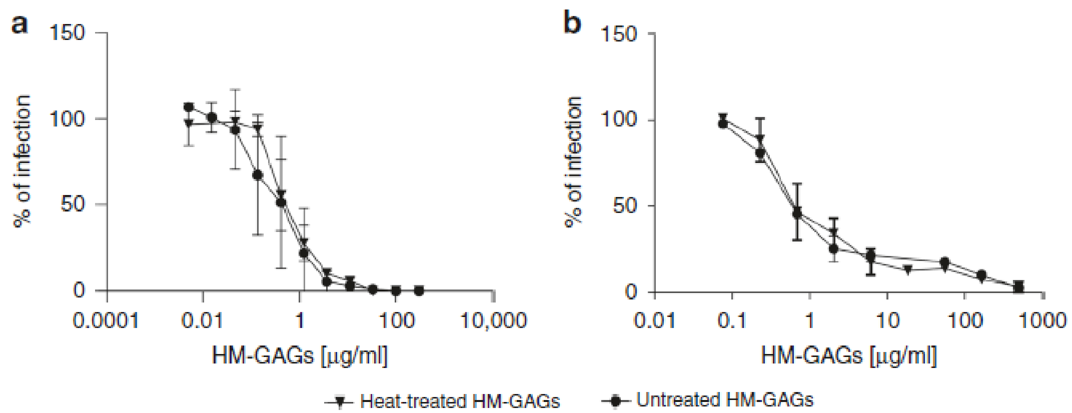

Figure 15: antiviral activity against HCMV (a) and RSV (b) of HM-GAGs after Holder pasteurization. Virus inhibition assays were performed by treating both HCMV (a) and RSV (b) with either heat-treated or untreated HM-GAGs during the cell penetration process. The dose-response curves illustrate the results, which are expressed as a percentage relative to the control (untreated virus). The data are shown as means ± SEM from three independent experiments, each conducted in triplicate. Statistical analysis using the F-test revealed no significant differences between the heat-treated and untreated groups (Francese, et al 2021).

5. Discussion

This research highlights the significant role of human milk (HM) in providing antiviral protection to infants, specifically through its glycosaminoglycan (GAG) components. While the antiviral effects of human milk have been documented against common pathogens of its effects against emerging flaviviruses, such as Zika virus (ZIKV) and Usutu virus (USUV), has been less prevalent in scientific literature.

This study, therefore, represents an important contribution to our understanding of the broad-spectrum antiviral properties of human milk across different stages of lactation and its specific bioactive components like GAGs.

Antiviral Activity of Human Milk Across Lactation Stages

A key finding of this study is the demonstration that human milk exhibits consistent antiviral activity against ZIKV and USUV throughout the stages of lactation—colostrum, transitional milk, and mature milk. This uniform activity challenges previous assumptions that early-stage milk, such as colostrum, would possess stronger antiviral properties due to its higher concentration of immune factors like secretory immunoglobulin A (sIgA) and other immune proteins (Newburg & Walker, 2007). While studies have shown colostrum to be particularly potent against viruses such as HCMV due to the presence of these immune factors (Newburg et al., 2005), our research suggests that the antiviral activity against ZIKV, and USUV is largely due to non-specific bioactive factors like GAGs, independent of maternal immunity.

This discovery indicates that the protective effects of human milk extend beyond the presence of maternal antibodies or previous viral exposures, suggesting a broader, intrinsic antiviral mechanism that functions consistently throughout lactation. The ability of human milk to protect against virus, irrespective of the stage of lactation, underscores the importance of breastfeeding for infant protection against viral infections, particularly in regions where these emerging viruses pose a growing threat.

Glycosaminoglycans (GAGs) in Human Milk and Their Role in Antiviral Protection

An important aspect of this research was the characterization of glycosaminoglycans (GAGs) present in human milk. The GAGs isolated from human milk were found to be primarily composed of low-sulfated chondroitin sulfate (CS), heparan sulfate (HS), and heparin (Hep), with trace amounts of dermatan sulfate (DS) and hyaluronic acid (HA).

Notably, the concentration of GAGs was highest in colostrum, particularly in preterm milk, and gradually decreased as lactation progressed.

These GAGs exhibited structural features, such as low charge density and specific sulfation patterns, that closely resemble endogenous cellular GAGs, which are often exploited by viruses as attachment factors (Newburg et al., 1998). The structural characterization revealed that approximately 55% of the HM-GAGs were composed of CS, while 40% consisted of low-sulfated heparin (FMHep). These findings are consistent with previous studies demonstrating that human milk contains a diverse array of GAGs, capable of interacting with viral particles and potentially inhibiting their binding to host cells (Clarke & Portelli, 2019).

A major focus of this research was the role of glycosaminoglycans (GAGs) in the antiviral activity of human milk. GAGs, such as chondroitin sulfate (CS) and heparan sulfate (HS), are known to play a role in viral inhibition by mimicking the cell surface receptors that many viruses use for entry into host cells. These GAGs act as soluble decoy receptors, binding to viral particles and preventing them from attaching to and infecting cells (Bartsch et al., 2020). This study confirmed that HM-GAGs are effective in inhibiting ZIKV, and USUV at physiological concentrations, particularly in the early stages of lactation when GAG levels are highest. This finding is consistent with prior research indicating that human milk components like HMOs and GAGs can block viral entry by interfering with viral binding to host cell receptors (Pichert et al., 2012).

The antiviral effect of GAGs is primarily seen in their ability to prevent the attachment of virus to host cells, a critical step in the viral replication cycle. Studies have shown that various viruses, including flaviviruses, utilize cellular GAGs as low-affinity receptors to concentrate the virus on the cell surface, thereby facilitating entry (Johansson et al., 2015). The GAGs in human milk appear to act by occupying these receptor sites, effectively blocking the virus from binding to host cells and establishing infection. This mechanism was particularly evident in our experiments where the GAGs from colostrum, the earliest form of milk, showed the strongest inhibitory effects.

Comparative Antiviral Properties Against Other Pediatric Viruses

Beyond ZIKV and USUV, this study also explored the antiviral potential of HM-GAGs against other viruses of pediatric concern, including HCMV, RSV, and human rotavirus (HRoV). The findings reveal significant antiviral activity of HM-GAGs against HCMV and RSV, with EC50 values that align with the physiological concentrations of GAGs in human milk, particularly in colostrum and early milk.

This antiviral action was seen to be selective, as no significant activity was observed against HRoV, which does not rely on GAGs or heparan sulfate proteoglycans (HSPGs) for cell entry (Newburg et al., 1998).

The selective antiviral effects highlight the specificity of HM-GAGs in targeting viruses that depend on GAGs for cellular attachment, such as RSV and HCMV. These findings are supported by previous research demonstrating the role of GAGs in inhibiting viruses that utilize heparan sulfate as an entry receptor (Clarke & Portelli, 2019). The fact that no antiviral activity was observed against HRoV underscores the need for further research to explore the broader implications of GAG structure in antiviral activity, as different viruses exploit different entry mechanisms.

Heat Stability and Practical Implications for Milk Banking

Another critical aspect of this study was the examination of the stability of HM-GAGs antiviral activity after heat treatment. Since donor milk is often subjected to pasteurization in human milk banks (HMBs), it was important to assess whether the antiviral properties of HM-GAGs were retained after thermal processing. This study demonstrated that Holder pasteurization (HoP), which involves heating milk to 63°C for 30 minutes, did not impair the antiviral efficacy of HM-GAGs against HCMV and RSV.

This finding has practical implications for milk banks, ensuring that pasteurized donor milk retains its protective antiviral properties, making it a safe alternative when maternal milk is unavailable.

The preservation of GAGs antiviral activity post-pasteurization is critical for infants, particularly preterm and immunocompromised infants who rely on donor milk from HMBs for nutrition and protection against infections. Previous studies have suggested that while pasteurization can reduce the concentration of immune proteins such as immunoglobulins and lactoferrin, it does not significantly affect the concentration or antiviral function of GAGs (Pichert et al., 2012).

Potential Therapeutic Applications and Future Research

The findings of this research open promising possibilities for the therapeutic use of GAGs as antiviral agents. Given their ability to inhibit viral attachment and replication, HM-GAGs could be further explored as potential therapeutic agents for preventing or treating viral infections in infants and other vulnerable populations. The idea of using GAGs as decoy

receptors to block viral entry offers a novel and natural approach to antiviral therapy, particularly in the face of emerging viral threats like ZIKV and USUV (Bartsch et al., 2020).

Moreover, the study raises important questions about the behavior of HM-GAGs after ingestion. While the evidence suggests that GAGs remain intact as they pass through the infant digestive system, more research is needed to understand whether they retain their antiviral properties throughout digestion and whether they exert protective effects beyond the gastrointestinal tract.

Further research should also explore the structure-specific interactions between GAGs and various viral pathogens. As demonstrated by the selectivity of HM-GAGs against certain viruses, the specific structure of GAGs—including their degree of sulfation and monosaccharide composition—appears to play a crucial role in their antiviral efficacy. Understanding these structure-function relationships could lead to the development of more targeted antiviral therapies based on GAGs.

Conclusion

In conclusion, this study provides compelling evidence of the intrinsic antiviral properties of human milk, particularly its glycosaminoglycan components. The ability of HM-GAGs to inhibit viral replication by preventing viral attachment to host cells highlights their importance in infant protection against a wide range of viral infections. The preservation of these antiviral properties after pasteurization further underscores the importance of human milk banks and the use of donor milk in neonatal care.

Moving forward, the potential therapeutic applications of GAGs in antiviral treatments deserve further exploration, particularly in the context of emerging viral threats and pediatric health.

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