Human milk glycosaminoglycans inhibit cytomegalovirus and respiratory syncytial virus infectivity by impairing cell binding

Human milk glycosaminoglycans inhibit cytomegalovirus and respiratory syncytial virus infectivity by impairing cell-binding

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Impact

- HM-GAGs have been poorly investigated for their antiviral action so far
- We demonstrated that HM-GAGs are endowed with significant anti-HCMV and anti-RSV activity and that they are able to alter virus binding to cell
- The contribution of individual HM-GAGs is mainly exerted by the FMHep and is not based on a simple charge interaction between virus and sulfate groups, but involves a specific GAG structural configuration
- Our results contribute to identifying the multiple factors synergically acting in mediating HM antiviral properties and to clarifying their specific mechanism of action

Abstract

Background: The antiviral role of glycosaminoglycans in human milk (HM-GAGs) has been poorly investigated. They are highly sulfated polysaccharides which were proposed to act as decoy receptors according to their structure. The aim of this study is to evaluate the antiviral potential and the mechanism of action of total and individual HM-GAGs against three pediatric clinically relevant viruses: respiratory syncytial virus (RSV), cytomegalovirus (HCMV) and rotavirus.

Methods: HM-GAGs were isolated from HM and a library of individual GAGs, structurally-related to HM-GAGs, was prepared. The antiviral activity of HM-GAGs and the impact of thermal treatment were investigated *in vitro* by specific antiviral assays.

Results: We demonstrated that HM-GAGs are endowed with anti-HCMV and anti-RSV activity and that they act by altering virus attachment to cell. We clarified the contribution of individual HM-GAGs, showing a specific structure-related activity. We did not observe any alteration of HM-GAGs antiviral activity after thermal-treatment.

Conclusion: We showed that HM-GAGs contribute to the overall antiviral activity of HM, likely exerting a synergic action with other HM antiviral agents. HM-GAGs can now be added to the list of endogenous factors that may reduce breast-milk-acquired HCMV symptomatic infections and protecting infants from respiratory tract infections by RSV.

Introduction

Human milk (HM) is a unique, extremely complex and highly variable biofluid that has evolved over millennia to nourish infants, support their growth and protect them from numerous diseases. It is a source of many macro and micro-nutrients for infant nutrition, but it also contains a myriad of biologically active components with profound role in infant survival and health [1–4]. A large body of literature has described several HM components conferring protection against viral pathogens [5,6] and, more recently, human milk glycosaminoglycans (HM-GAGs) have been added to the list [7–9]. HM-GAGs are highly sulfated, complex, linear polysaccharides constituted by repeating disaccharidic units, which are present in HM as a complex mixture made up of chondroitin sulfate (CS)/dermatan sulfate (DS), heparan sulfate (HS)/heparin (Hep) and a minor percentage of hyaluronic acid (HA), with the CS/DS fraction being the most represented (~ 55%) followed by HS/Hep (~ 40%) [10]. Coppa et al.[10] evaluated total and single GAGs species in human milk: a low-sulfated CS mainly formed of glucuronic acid (~96%) with a very low charge density of ~0.36 and no DS were detected and fast moving (FM)Hep/HS with lower sulfation (~2.00) than normal Hep were also observed in this biofluid. Quantitative analyses performed on HM from healthy term mothers showed the presence of 400 µg/ml of total GAGs at 30-45 days post-partum with 230 µg/ml of low-sulfated CS, 165 µg/ml of FMHep/HS and ~5 µg/ml of HA.

Since glycosaminoglycans (GAGs), with their great number of sulfate and carboxyl groups, represent common cellular receptors for a wide range of viral pathogens, HM-GAGs could act as decoy receptors, directly interacting with viruses and preventing their attachment to cells [11–13]. To explore this feature, we investigated the antiviral properties of HM-GAGs against a panel of viruses clinically relevant for infants: the human cytomegalovirus (HCMV), responsible for the most common congenital infection affecting approximately 1 out of every 150 live-born infants [14,15], the respiratory syncytial virus (RSV), representing the leading cause of lower

respiratory tract infections in children worldwide [16,17] and the human rotavirus (HRoV), the main cause of severe gastroenteritis in children up to five years old [18]. In particular, HCMV is shed into HM by almost every seropositive woman, with maternal HCMV reactivation that can already be detected in the colostrum and normally ends about three months after birth [19,20]. Nevertheless, systematic reviews have shown that breast milk-acquired HCMV infection with symptomatic disease is relatively rare[21,22], a likely consequence of the protective effects of HM components against viral infections. Indeed, the intrinsic antiviral action of HM against different viruses has been previously established [23–25], with different HM compounds such as immunoglobulins, lactoferrin, lactadherine, oligosaccharides (HMOs), extracellular vesicles (EVs) and others involved in this activity [23,25–28]. Nevertheless, the totality of factors synergically acting in mediating the HM antiviral action, along with the contribution of individual compounds and their mechanism of action remain partially unexplored [5].

Here we report, for the first time, that HM-GAGs are endowed with significant antiviral activity against HCMV and RSV and that they act by altering virus binding to the host cell. We clarified the contribution of individual HM-GAGs (by testing purified GAGs of various nature, structure, characteristics and content) to the overall antiviral activity of these components and we demonstrated that HM-Hep exerts the predominant action.

Methods

HM-GAGs isolation and characterization

HM samples were obtained from the Human Milk Bank (HMB) of the Città della Salute e della Scienza of Turin, Italy. An ethical review process was not required for this study. Each milk donor signed a written consent form, where mother's and infant's data protection was assured.. The donors cleaned their hands and breasts according to the Italian HMB guidelines [29], and the milk samples were collected in sterile bisphenol-free polypropylene bottles using a breast pump and immediately stored at -20˚C. HM-GAGs were extracted from a unique pool (final volume 50 ml) of mature milk (MM) collected from three mothers at 2 months after partum. The GAGs isolation procedure is described in the Supplementary Materials in details.

Total and single GAGs content and the main structural parameters of the single species were determined according to the methodology already illustrated in the previous study[8]. The procedure is briefly described in the Supplementary Materials.

GAGs library

Highly purified GAGs of various nature having different structure, characteristics and physico-chemical properties were tested for their possible anti-HCMV and anti-RSV capacities in an effort to clarify a structure-activity relationship useful to clarify the HM-GAGs properties. Table I illustrates the different GAGs used in this study and their main characteristics. More details are reported in the Supplementary Materials.

Heat treatment of HM-GAGs pool

HM-GAGs thermal treatment was performed in a PCR-thermocycler (C1000 Thermal Cycler, Bio-Rad), incubating 100 µl of HM-GAGs at 63°C for 30 min and then rapidly cooling at 4°C. As control, an equal volume of PBS was treated under the same conditions. The antiviral

activity of the heat-treated GAGs was determined by the virus inhibition assay, described below, and compared with the activity of the untreated samples.

Cell lines and viruses

Human foreskin fibroblast (HFF-1; ATCC SCRC-1041), Hep-2 human epithelial cells (ATCC® CCL-23) and African green monkey kidney epithelial cells (MA104; ATCC® CRL-2378.1) were grown as previously described [25,30,31]. .

The HCMV Towne strain incorporating the GFP sequence and the HCMV AD169 laboratory strain (ATCC VR-538) were propagated on HFF-1 cells[30]; the RSV strain A2 (ATCC VR-1540) was propagated on Hep-2 cells[32]. The human rotavirus (HRoV) strain Wa (ATCC® VR-2018) was activated with 5 μg/ml of porcine pancreatic trypsin type IX (Sigma-Aldrich) for 30 min at 37 °C and propagated in MA104 cells using DMEM containing 0.5 μg of trypsin per ml [33]. Viral stocks production and titration procedures are reported in Supplementary Materials.

Virus inhibition assays

HFF-1, Hep-2 and MA104 cells were seeded in 96-well plates. The antiviral assays were performed by incubating serial dilutions of HM-GAGs (HM-GAGs pool or individual compound) with virus (MOIs of 0.02 for HCMVs and HRoV, MOI of 0.01 for RSV) for 1h at 37°C. The mixtures then were added to HFF-1 cells for 2 h, to Hep-2 cells for 3 h or to MA104 cells for 1h for HCMV, RSV and HRoV respectively. The inocula were removed and the cells were washed twice. HCMV and RSV infected cells were overlaid with a 1.2% methylcellulose 2% FBS DMEM medium and fresh medium was added to HRoV infected cells. After 5 days, the HCMV Towne-infected green fibroblasts were visualized by fluorescence microscopy and counted.. The HCMV AD169 and the RSV infected cells were fixed with cold methanol-acetone (1:1) and subjected to indirect immunostaining after 5 or 3 days, respectively. The

HRoV infected cells were fixed after 16 h of incubation and subjected to virus-specific immunostaining. The immunostained cells and syncytia were counted. The effective concentrations that produced a 50% reduction of virus infection (EC_{50}) were determined by comparing the treated with the untreated wells.

Viability assay

Cell viability was determined using the MTS assay as previously described [8]. The 50% cytotoxic concentrations (CC_{50}) were determined. The procedure is described in the Supplementary Materials in detail.

Virus inactivation assay

150000 FFU of HCMV-T or RSV were challenged with HM-GAGs (80 µg/ml or 400 µg/ml) in a final volume of 200 µl for 1h at 37˚C. As control, 150000 FFU were incubated with culture medium supplemented with an equal volume of PBS 1X. The treated and untreated viruses were then titrated on confluent cells to the non-inhibitory dilution of HM-GAGs. The residual viral infectivity was determined by means of fluorescence microscopy (HCMV-T) or by indirect immunostaining (RSV) after 5 days or 3 days respectively.

Binding assay

HFF-1 and Hep-2 cellsseeded in 24 well plates and viruses (HCMV-T or RSV, MOI = 3) were cooled to 4˚C for 10 minutes. The viruses were then allowed to bind cells in the presence of HM-GAGs (HM-GAGs pool or single compound) at concentrations of 80 µg/ml, 400 µg/ml or 2500 µg/ml unless otherwise stated. After 1 h on ice at +4 °C, the cells were washed twice to remove any unbound virus andsubjected to three rounds of freeze-thawing to release the bound viral particles. The lysates were clarified by means of low-speed centrifugation. The cell-bound

virus titers were determined by means of titration on confluent cells followed by fluorescence microscopy observation (HCMV-T) or indirect immunostaining (RSV).

Data analysis

All the results are presented as the mean values of three independent experiments. The EC_{50} values of the inhibition curves were calculated by a regression analysis using GraphPad Prism software, version 8.0 (GraphPad Software, San Diego, CA) by fitting a variable slope-sigmoidal dose-response curve. Statistical analysis was performed using Student's test, ANOVA Analysis of variance or the F-test, as reported in the figure legends..

Results

Anti-HCMV and anti-RSV activity of HM-GAGs

A pool of GAGs was isolated from mature HM samples (HM-GAGs) from healthy donor mothers and subsequently characterized and tested *in vitro*. According to the previous study[8], the HM-GAGs extract further tested in this new study was mainly composed of a low-sulfated CS (~55%) and of a low sulfated Hep also known as FMHep (~40%) with trace amounts of DS, high-sulfated Hep and HA (Figure S1). The charge density of the low-sulfated CS detected in the HM-GAGs extract was 0.33, whereas the reported charge density values of natural CS samples are generally greater than 0.90 and 0.94 for CS purified from bovine milk [34,35]. Moreover, the low-sulfated CS showed a ratio of sulfated groups in position 4 and 6 of *N*-acetyl-galactosamine of 1.75. Additionally, the FMHep component of HM-GAGs mixture had a charge density of 2.05, in contrast to an average charge density value of the main natural Hep samples of ~2.40-2.90 [36] and of the Hep purified from bovine milk of 2.28 [10].

The first set of antiviral experiments was performed to investigate the activity of a HM-GAGs pool against three viruses with high clinical relevance for infants: HCMV, RSV and HRoV. Briefly, the virus inhibition assay was performed by treating viruses with HM-GAGs before and during the cell-penetration process, as reported in Materials and Methods section. We demonstrated that HM-GAGs exerted a net antiviral activity against two different laboratory HCMV strains (AD169 and Towne HCMV) and RSV with EC_{50} values in the range of sub-physiological concentrations (from 0.37 to 1.89 μg/ml), whereas no antiviral activity was observed against HRoV (Table II and Figure S2). Moreover, in order to exclude the possibility that the observed antiviral action was due to cytotoxicity, viability assays were performed by treating cells with HM-GAGs under the same experimental conditions as the virus inhibition assay. Results revealed that HM-GAGs were not toxic for cells even at the highest tested

concentration (1500 µg/ml), thus resulting in favorable selectivity indexes (CC_{50}/EC_{50}) for the prosecution of the study.

HM-GAGs alter the binding of HCMV and RSV to the host cell

The second set of antiviral experiments was addressed to investigate the mechanism of the antiviral action of HM-GAGs. Taking into account the structural similarity of HM-GAGs with cellular receptors and the experimental conditions whereby they were initially tested, we hypothesized two different mechanisms of action: the direct impairment of viral particle or the alteration of virus binding to the host cell. Therefore, we first investigated whether HM-GAGs were endowed with virucidal activity by performing virus inactivation assays. We tested two different HM-GAGs concentrations: 80 µg/ml, an effective high concentration corresponding to the highest EC_{99} value within the 95% CI and 400 µg/ml, corresponding to the lowest HM-GAGs physiological concentration detected in mature HM. As shown in Figure 1A and 1B, HCMV (Towne strain) and RSV pre-incubation with HM-GAGs did not affect viral infectivity. On the contrary, when the same HM-GAGs concentrations were added to cells simultaneously with viral particles at 4°C for 1h (i.e. binding assay), the titers of HMCV and RSV bound to cells were reduced in a dose-dependent manner (Figure 1C and 1D) indicating that HM-GAGs act by preventing the binding of both viruses to cells. Numerical results of virus inactivation assays and binding assays are reported in Table S1 and S2 respectively (Supplementary Materials).

Anti-HCMV and anti-RSV activity of a HM-GAGs library

Prompted by the above findings, we sought to investigate the contribution of single polysaccharides to the overall antiviral activity of HM-GAGs. To this aim, a library of 12 GAGs

(see Table I) was screened against HCMV and RSV through the virus inhibition assay and their possible cytotoxicity was also investigated on HFF-1 and Hep-2 cells.

As reported in Table III and Table IV, FMHep resulted the most effective GAG against both viruses, with EC_{50} values corresponding to 0.09 µg/ml for HCMV and 0.99 µg/ml for RSV. Furthermore, even to a less extent, CS1, chondroitin and DS showed considerable antiviral activities against both viruses, with EC_{50} values ranging from 22.75 µg/ml to 1729 µg/ml. Additionally, CS5 was slightly active against RSV (EC_{50} = 1582 µg/ml). Interestingly, not all CS samples as well as the four HA products with different molecular weight exerted antiviral activities, demonstrating structure-related biological properties.

No polysaccharide was toxic for cells: as expected, the CC_{50} values were greater than 5000 µg/ml for all the tested molecules, confirming that the observed antiviral activity was not due to an alteration of cell viability.

After identifying the HM-GAGs endowed with anti-HCMV and anti-RSV activity, we investigated their ability to inhibit viral binding to the host cell. As reported in Figure 2A, CS1, chondroitin and DS significantly inhibited HCMV binding when tested at 2500 µg/ml. FMHep showed the highest inhibitory activity: the concentration of 100 µg/ml reduced the titer of HCMV bound to cells of more than 1 Log. CS1, chondroitin and DS (at concentration of 2500 µg/ml) also markedly altered RSV binding to Hep-2 cells, but we did not observe a significant RSV inhibition by CS5. Consistently with the results obtained with HCMV, FMHep resulted the most active GAG in inhibiting RSV binding: 100 µg/ml concentration was enough to reach a titer reduction greater than one order of magnitude and comparable with the one obtained with the total HM-GAG preparation (Figure 2B). Numerical results of binding assays are reported in Table S3 (Supplementary Material). Altogether, these results indicated that individual HM-GAGs may act synergically to alter HCMV and RSV binding to cells, with FMHep exerting the predominant action.

The antiviral activity of HM-GAGs is maintained after heat treatment

Finally, since donor milk from HMBs is considered the best alternative when mothers' own milk is unavailable, we investigated the impact of Holder pasteurization (HoP), the commonly used pasteurization method, on HM-GAGs antiviral activity. We treated HM-GAGs preparation under laboratory simulated conditions of HoP (63°C for 30 min) and tested the residual antiviral activity. As reported in Figure 3A and 3B, the antiviral activity of the HM-GAGs was maintained after heat treatment. The EC_{50} values of heat-treated and untreated HM-GAGs were comparable for both viruses, suggesting that HoP may preserve the anti-HCMV and anti-RSV activities of the compounds.

Discussion

In recent years, numerous components of HM have been investigated for their antiviral properties [5,6]. Among them, HMOs, which represent the third largest class of components within HM after lactose and lipids, were demonstrated to mimic structures of viral receptors, thus blocking adherence to target cells and preventing infection [27,37]. On the contrary, the other major family of complex carbohydrates of HM, the HM-GAGs, has been poorly investigated with only a few reports indicating their antiviral potential [7,8,38]. To reply to this lack of knowledge, we investigated the protective properties of HM-GAGs against three viruses of pediatric clinical relevance, namely HCMV, RSV and HRoV. The first notable finding was that the complex mixture of HM-GAGs was endowed with net anti-HCMV and anti-RSV activity *in vitro* and that it was able to significantly impair virus binding to cells. No antiviral activity was observed against HRoV instead. This selective activity, along with the mechanism of action, could be explained with HCMV and RSV dependence on heparin sulfate proteoglycans (HSPGs) for attachment to cells [39–41]. The lack of anti- HRoV activity is consistent with this hypothesis since HRoV does not exploit HSPGs as attachment receptors [42]. Indeed, HSPGs are composed by a core protein covalently linked to heavily sulfated GAGs chains, which can result in competition with HM-GAGs for the binding to the viral particles. In other words, HM-GAGs can act as decoy receptors, i.e. glycominetics, binding the viral particles and displacing the link with cellular GAGs. The lack of virucidal activity, demonstrated by means of virus inhibition assays, indicated that the link between HM-GAGs and HCMV or RSV is presumably transient and not able to permanently inactivate the viral particles before cell infection. The simultaneous presence at cell surface level of HM-GAGs and viruses is necessary for the antiviral activity to occur.

All the experiments were performed at concentrations that would be relevant to breastfed infants. In particular, the literature reports that the highest GAGs amounts are present at 4 days

after partum (~ 9300 μg/ml and ~ 3800 μg/ml in preterm and term milk, respectively), followed by a progressive decrease up to day 30 (~ 4300 μg/ml and ~ 400 μg/ml, respectively) [43]. Herein, we reported that the EC_{50} values of HM-GAGs against both HCMV and RSV are about two orders of magnitude lower than the concentration in mature HM. Additionally, we showed that the binding inhibition is still exerted at sub-physiological concentration (80 μg/ml), reaching the highest activity at 400 μg/ml. Since the inhibition of HCMV and RSV binding is not complete, we may conclude that HM-GAGs only partially contribute to the overall antiviral activity of HM, likely exerting a synergic action with other antiviral compounds detectable in this rich biofluid. To the best of our knowledge, this is the first study reporting the antiviral potential and the mechanism of action of HM-GAGs against the above-mentioned viruses. There are only other two available studies on the topic. Viveros-Rogel M. *et al.* demonstrated that HM-GAGs are slightly active against HIV-1 at high concentrations in peripheral blood mononuclear cells (PBMC) [38] and Francese R. *et al.* showed that HM-GAGs are active against two emerging flaviviruses, zika virus (ZIKV) and usutu virus (USUV), in the range of physiological concentrations detected in term and preterm human colostrum [8].

In the second part of the study, we investigated the anti-HCMV and anti-RSV properties of a library of individual GAGs. Despite several reports showed that GAGs of different origins and HSPG-mimicking compounds represent broad-spectrum antiviral agents [44–49], a small number of studies investigated the antiviral properties of GAGs in HM. Since HM-GAGs are mainly represented by low-sulfated CS, low-sulfated Hep or HS and HA, we individually tested different kinds of CS, a low-sulfated Hep known as FMHep and four samples of HA differing for their molecular weight. We demonstrated the antiviral activity of three exogenous GAG samples: the CS1 composed of GlcUA and having charge density greater than 0.94 and a 4s/6s ratio of 1.67, the non-sulfated chondroitin and the low-sulfated Hep with charge density of 2.51 and constituted of a high percentage of IdoUA (see Table I). It is worth of mention that these

three GAGs strictly resemble the structure and properties of endogenous HM-GAGs. CS1 is a highly sulfated CS with a 4s/6s ratio very close to that of HM-CS that is 1.75. On the other hand, the other three tested CS (CS2, CS3 and CS4) are ineffective and CS5 with a 4s/6s ratio of 1.81 shows only a partial anti-RSV activity. The non-sulfated chondroitin showing anti-HCMV and anti-RSV capacities is very close to the HM-CS component which is a low-sulfated CS with a very low charge density of 0.33. Overall, we observed that the main parameters conferring antiviral activities to CS are the reduced number of sulfate groups per disaccharide units and/or, when present, a ratio of these groups in position 4 about double than those in position 6 of N-acetyl-galactosamine. Interestingly, this structure-related antiviral activity is highly specific. In fact, HA of various molecular weight constituted of 100% GlcUA, with N-acetyl-glucosamine and not N-acetyl-galactosamine as in CS, shows no activity. The other abundant HM-GAG component is a low-sulfated Hep (charge density of 2.05) known as FMHep which is very close to our exogenous polymer possessing a quite similar charge density of 2.51. We showed also for this heteropolysaccharide a strict structure-activity relationship and consistently with previous studies [44–49] reporting the antiviral activity of Hep and or HS, we indicated that FMHep is the most effective GAG. Finally, even if present in trace amounts in HM, we also tested the antiviral activity of a DS sample that is quite exclusively constituted of IdoUA and N-acetyl-galactosamine and mainly sulfated in position 4 of this last monosaccharide (see Table I). We observed a discrete anti-HCMV and anti-RSV activity that is coherent with some previous studies showing that DS (chondroitin sulfate B) was slightly active against RSV *in vitro*, but not against HCMV. Portelli et al.[50] reported that the concentrations of DS required to significantly inhibit RSV were in excess of those found in HM and would not be expected to have any anti-RSV activity during breastfeeding. Moreover, a slight or no effect was measured for CS samples but no structural characterization of these GAGs is available. In another study, Clarke et al.[51] tested various kinds of CS (CSA, CSC and

DS) against a preterm milk-isolated and a clinical isolate HCMV strain and against three different types of rhinoviruses. They also did not observe any significant antiviral action against all the virus strains but, on the contrary, DS was found to slightly favor the milk-borne HCMV strain infection. The antiviral capacities of DS and FMHep may be due to the presence in their backbones of the IdoUA component [52], but we further demonstrated the activity of CS with peculiar characteristics related to a low charge density and to sulfate groups in specific positions of the polysaccharide chains compatible with the GAGs present in HM. Our results therefore indicated that the role of HM-GAGs in anti-RSV and anti-HCMV activity is not based on a simple charge interaction between virus and sulfate groups, but involves a specific GAG structural configuration that includes IdoUA and sulfate groups for FMHep or HS and a minimum of sulfate groups in specific position for a low-sulfated CS.

As regards the mechanism of action, all the identified active GAGs were able to reduce the titer of HCMV and RSV bound to cells. Nevertheless, the binding inhibition was mainly mediated by HM-FMHep, which was active already at low concentrations (100 µg/ml). Consistently with our results, Newburg *et al.* previously found that multiple forms of HM-GAGs inhibit the ability of the HIV envelope glycoprotein (gp120) to bind the host cell receptor (CD4), but in this case the binding inhibition was mainly related to a CS or CS-like moiety [7]. Altogether our findings suggested that CS1 (CS5 only for RSV), Chondroitin and FMHep may all synergically contribute to the anti-HCMV and anti-RSV activity of HM-GAGs, although to a different extent with FMHep being the most effective.

An open question is whether HM-GAGs can still exert their antiviral action after ingestion. Interestingly, it was previously demonstrated that the elevated content of HM-GAGs passes undigested through the entire digestive system of newborns. Once reaching the colon, these complex macromolecules are catabolized by some bacterial enzymes and monosaccharides/oligosaccharides are utilized for bacterial metabolism or internalized by

intestinal cells [53]. We can therefore speculate that since HM-GAGs can reach the intestinal lumen remaining intact, they could exert their antiviral action as soluble receptors, throughout all the digestive tract and, after being catabolized and internalized, they may also have a still unknown protective role at distant body sites.

Finally, since donor milk is an important nutritional alternative when mother's own milk is unavailable, we investigated whether HoP, a common practice in HMBs [54,55], could affect the antiviral potency of HM-GAGs. Indeed, HoP guarantee the microbiological safety of donor milk, but it is known to alter some of the nutritional and biological properties of this biofluid, in particular some specific proteins with immunologic and anti-infective action (such as immunoglobulins and lactoferrin) [56]. Nonetheless, previous studies demonstrated that HoP does not affect HM-GAGs content and pattern: the relative percentages of FMHep (or HS) and CS along with the main parameters related to GAG structure were maintained in pasteurized-HM compared to untreated controls [57]. As presumed, we found that also the antiviral properties of HM-GAGs were maintained after heat treatment, indicating that HoP does not alter the anti-HCMV and anti-RSV activities of these compounds. Despite this evidence, it should be noted that we used an HoP-like thermal process that consisted in treating just a small volume of already extracted HM-GAGs at 63°C for 30min. Our results cannot consequently be directly applied to HMBs and should be confirmed on HM-GAGs extracted from HM pools pasteurized in real working conditions.

In conclusion, our results contribute to identifying the multiple factors synergically acting in mediating HM antiviral action and to clarifying the biological roles of such abundant components as HM-GAGs. These compounds can now be added to the list of endogenous factors that may play a role in reducing breast-milk-acquired HCMV symptomatic infections and in protecting the breastfed infant from respiratory infections by RSV..

Study limitations: With the aim to explore the antiviral activity of HM-GAGs, we first isolated these components from a pool of MM samples. We chose this specific maturation stage because it is the most bioavailable one. However, we cannot exclude possible changes in HM-GAGs antiviral activity when isolated from colostrum or transitional milk. Indeed, a recent study indicated that along with the reduction of HM-GAGs total amount over lactation, also minimal changes in terms of HM-GAGs quality and pattern are detectable. More precisely, HS exhibits a slight increase in the degree of sulfation and the trend of CS sulfation first increases and then decreases[58]. Therefore, it would be interesting to explore the antiviral potential of HM-GAGs extracted from different milk maturation stages and from a larger cohort of donor mothers, ideally subdivided according to their biological characteristics and medical history.

A further limitation of this study is that the experiments were conducted *in vitro*. Therefore, it remains to be investigated whether HM-GAGs can play a protective role *in vivo* by preventing the development of clinically relevant viral infections in the infant. Further studies are needed to understand the antiviral role of HM-GAGs also in synergy with other protective factors present in HM.

Data availability statement: All data generated or analysed during this study are included in this published article.

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Figure Legends

Figure 1: Virus inactivation assay (A and B) and binding assay (C and D). In the virus inactivation assay, HCMV-T (A) or RSV (B) were incubated with HM-GAGs (80 or 400 µg/ml) for 1h at 37°C and subsequently the residual viral infectivity was evaluated by titration to the non-inhibitory dilution of HM-GAGs. For the binding assay, HCMV-T (C) or RSV (D) (MOI=3) were allowed to attach to cells in presence of HM-GAGs (80 or 400 µg/ml) for 1h on ice. The cell-bound virus titers were then determined by means of titration on confluent cells. On the y-axis, the infectious titers are expressed as focus-forming unit per ml (FFU/ml) (A, C) or plaque-forming units per ml (PFU/ml) (B, D). Error bars represent standard error of the mean of three independent experiments (ANOVA and Bonferroni Post hoc test; *** $p < 0.001$; ns: not significant).

Figure 2: Binding assay with individual GAGs. HCMV-T (A) or RSV (B) (MOI=3) were allowed to attach to cells in presence of individual GAGs (CS1, chondroitin, CS5, DS: 2500 µg/ml, FMHep: 100 µg/ml) for 1h on ice. The cell-bound virus titers were then determined by means of titration on confluent cells. On the y-axis, the infectious titers are expressed as focus-forming unit per ml (FFU/ml) (A) or plaque-forming units per ml (PFU/ml) (B). Error bars represent standard error of the mean of three independent experiments (ANOVA and Bonferroni Post hoc test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns: not significant).

Figure 3: Anti-HCMV (A) and anti-RSV (B) activity of HM-GAGs after heat treatment at 63°C for 30 min. HM-GAGs thermal treatment was performed by incubating 100 µl of HM-GAGs at 63°C for 30 min and then rapidly cooling at 4°C. The virus inhibition assay was performed by treating HCMV-T (A) or RSV (B) with heat-treated or untreated HM-GAGs before and during the cell-penetration process. The dose-response curves are reported. Data are presented as percentage of control (untreated virus). Values are reported as means ± SEM of

three independent experiments performed in triplicate. The EC_{50} values of heat-treated and untreated HM-GAGs were determined (respectively 1.03 and 0.79 µg/ml for RSV, 0.58 and 0.37 µg/ml for HCMV). Data were compared by F-test showing no significant difference.

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