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Impact of holder pasteurization on protein and eNAMPT/Visfatin content in human breast milk

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Human milk proteins, a mixture of whey proteins including caseins, milk fat globule membrane (MFGM) proteins, various peptides, and their amino acids, play a crucial role in infant growth and development, as do non-nutritional bioactive components. The extracellular nicotinamide phosphoribosyltransferase (eNAMPT) or visfatin is a conserved cytokine/enzyme released by many mammalian cells, related to multiple metabolic and immune processes. Few investigations have been reported about detecting visfatin in skimmed milk and the hypothesis of its potential role in regulating infant adiposity through breast milk. Milk samples from a donated human milk bank were analyzed. After milk fractionation by centrifugation, skimmed milk and MFGM were analyzed by SDS-PAGE, MALDI-TOF mass spectrometry ELISA and/or Western blot. The ELISA assay showed a higher visfatin content in raw skimmed milk than in pasteurized samples. Meanwhile, MFGMs revealed higher visfatin levels in pasteurized samples. This is the first time visfatin has been identified associated with MFGM, and these results could suggest an affinity of this molecule for a lipidic environment.

Keywords Human breast milk, Holder pasteurization, Visfatin, eNAMPT, ELISA, Western blot

Maternal milk is considered the optimal source of nutrition for infants across species due to its comprehensive profile and health benefits. The World Health Organization (WHO) substantiates this assertion by recommending exclusive breastfeeding from birth through the first six months of life, after which to use it as a complement to the diet until two years of age[1]. The composition of human milk is the result of millions of years of evolution: it comprises the perfect amount of water, proteins, lipids, carbohydrates, vitamins, and micronutrients, i.e., all the nutrients that infants need until six months of age when it is necessary to implement the diet with complementary foods[2]. Indeed, human milk can be described as a dynamic and vital tissue in which the components vary in quantity according to the infant's needs between feedings and throughout the lactation period[3]. Human milk proteins are a mixture of whey, caseins, and various peptides: their amino acids play a crucial role in the growth and development of the child[4]. Some of these proteins, such as α-lactalbumin, lactoferrin, and lysozyme, are also important for infants as non-nutritional bioactive components[5]. Moreover, human milk contains antibodies (mainly IgA), cytokines, such as interferon-γ, and transforming growth factor-β, providing immunomodulation and passive protection to the baby[6]. In addition, the protein extracellular nicotinamide phosphoribosyltransferase (eNAMPT), also known as visfatin[7,8], is present in human milk[9]. Visfatin, or pre-B-cell colony-enhancing factor (PBEF) or NAMPT, is a 52 kDa protein secreted from the visceral adipose tissue, and it is correlated with several metabolic and immune processes[7,8,10–12]. The presence of visfatin in human milk may imply its potential role in regulating infant adiposity through maternal milk[9]. Yoonezawa and colleagues proposed that eNAMPT/visfatin plays an important role in the bovine lactating mammary gland and that it could be secreted by these epithelial cells[13]. Another study assessed the eNAMPT/visfatin levels in serum and breast milk of mothers of preterm infants, suggesting that this molecule could protect them from weight loss after birth[11].

Since human breast milk is the recommended choice for feeding a newborn baby, it is essential for those infants who cannot be breastfed to be fed with donated human milk (DHM)[14]. DMH is collected in a Donated Human Milk Bank (DHMB), following local legislation, from healthy lactating women, and it is used to feed children hospitalized in neonatal intensive care. Immediately after donation, DHM undergoes the Holder pasteurization process to inactive pathogens, reduce bacterial contamination, and stabilize the milk[15]. While

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Holder pasteurization remains the recommended method according to international guidelines for establishing Human Milk Banks, ongoing discussion surrounds the impact of this pasteurization process on protein content[16–19].

In this work, we aim (i) to evaluate the effect of Holder pasteurization on the protein content and profile of donated human milk, (ii) to detect visfatin in both skimmed milk and MFGM-associated proteins, and (iii) to determine if there are differences in visfatin content between pasteurized and non-pasteurized human breast milk.

Results

Milk samples

In this study, we investigated human milk samples kindly provided by DHMB of Ospedale Sant'Andrea, Vercelli, Italy. Between January and December 2023, 10 milk samples were collected from 10 healthy lactating mothers. All pregnant mothers, except for donor 1 who had a cesarean section, delivered by vaginal delivery. All milk samples were mature and donated at least five weeks after delivery. Sample 10 was excluded from the subsequent analysis because, in the pasteurized version, the skimmed milk fraction could not be separated from the fat globule fraction. Its protein concentration and visfatin quantification range in the same interval as the other samples. Supplementary Table 1 reports the demographic characteristics of milk donors.

Protein quantification

Figure 1A reports the protein concentration of milk samples before (NP) and after Holder pasteurization (P). Four samples of skimmed milk show a statistically significant difference between the treated and raw samples, with a higher protein concentration in the NP ones. The MFGM-associated proteins exhibit an inverse trend. Except for samples 1 and 9, all samples subjected to the Holder pasteurization process demonstrate a higher protein concentration, as illustrated in Fig. 1C. Moreover, by analyzing the samples as a cohort, it is possible to identify a significant trend of decreasing protein content of skimmed milk (Fig. 1B) and increasing content of protein associated with fat globule membranes (Fig. 1D).

Protein profiling

The SDS-PAGE profiles of pasteurized MFGM-associated proteins appear to have some more intense bands around 250, 150, and 75 kDa, in addition to those between 50 and 37 kDa and between 37 and 25 kDa (Supplementary Fig. 1). The profile does not show the appearance or disappearance of any new bands. For skimmed milk proteins, the intensity of the bands is almost unchanged between the samples. The very intense 75 kDa band corresponds to lactoferrin, while the thinner band below corresponds to serum albumin and immunoglobulin heavy chain. Just above the 25 kDa band is β-casein, as reported in our previous work[20]. An aliquot of each skimmed milk sample was subjected to MALDI-TOF analysis to identify low molecular weight proteins. Upon examination of the spectra (Supplementary Figs. 2 and 3), discernible distinctions between not pasteurized and pasteurized samples are not evident. In both not pasteurized and pasteurized DHM samples analyzed with the linear positive method, some peaks are visible, around 24,000 m/z, 14,000 m/z, 12,000 m/z, 8,000 m/z, 7,000 m/z, and the 4,000–5,000 m/z range. Information about m/z values and peak intensity was submitted to the GeenaR online platform to investigate differences in the molecular weight distribution of skimmed milk DHM samples. The principal component analysis has confirmed the hypothesis since it has not identified a discriminant between the pasteurization and the not pasteurized samples.

eNAMPT/visfatin detection

An ELISA assay quantified the eNAMPT/visfatin concentration within the skimmed milk samples. With only one exception (Fig. 2A), the trend demonstrates decreased eNAMPT/visfatin levels after the pasteurization process. The difference is statistically significant in four out of nine sample pairs analyzed. The medium concentration of eNAMPT/visfatin found in skimmed milk is 1,104.711 ng/ml in not pasteurized samples, while in pasteurized ones, the value decreased to 540.230 ng/ml. These values are higher than those usually reported in blood for eNAMPT[9,21].

Anti-eNAMPT/visfatin immunoreactive bands showed higher intensity in pasteurized MFGM samples than in not pasteurized milk. The bands that reacted are just above 50 kDa (Fig. 2C), consistent with the presence of eNAMPT/visfatin, whose molecular weight is around 52 kDa. Sample 2, which has small amounts of visfatin, also follows the same trend, showing a slight band of reactivity in the pasteurized version compared to the unpasteurized one.

To confirm the observations, an optical density analysis of these bands was performed (Fig. 2D and Supplementary Fig. 4). The differential distribution of eNAMPT/visfatin between P and NP samples has been assessed by the recombinant protein (Supplementary Fig. 5).

The cohort study shows that the eNAMPT/visfatin expression follows the same trend as the overall protein concentration, decreasing in pasteurized skimmed milk samples and increasing in pasteurized MFGM samples (Figs. 1B and D and 2B and D).

Discussion

The research proposed in this paper adds a pillar to the study of Holder pasteurization's effect on the protein concentration in human milk before and after treatment. Holder pasteurization carried out at 62.5 °C for 30 min, is presently considered the gold standard for milk processing in human milk banks. It is endorsed by numerous guidelines as the best balance between maintaining milk quality and ensuring microbiological safety[22,23]. Conflicting results are reported in the literature concerning the decrease or increase in protein

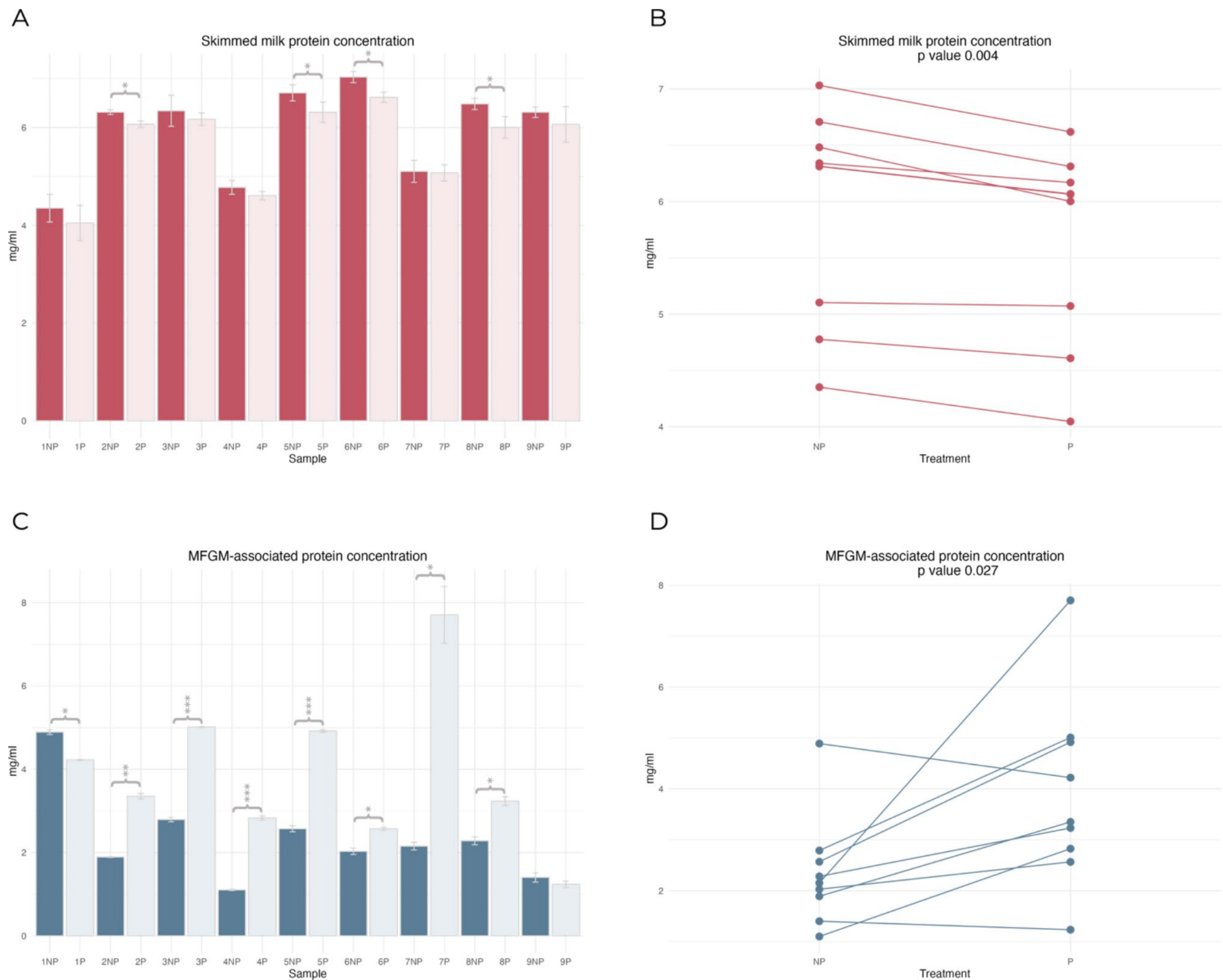

Fig. 1. Analysis of protein concentration. Protein concentration data of skimmed milk (**A**, **B**) and milk fat globule membrane-associated protein (**C**, **D**) before (NP) and after Holder pasteurization (P). The data presented in (**A** and **C**) are expressed in mg/ml and presented as the mean protein concentration, derived from three technical replicates ± standard deviation. Additionally, statistical significance, calculated with a t-test, is indicated by "***" (p-value < 0.001), "**" (p-value < 0.01), and "*" (p-value < 0.05). (**B** and **D**) represent the data studied as a cohort; statistical analysis was performed with the Wilcoxon matched-pairs signed rank test.

caused by the pasteurization process[19,24–27]. Specifically, this study examined two phases: skimmed milk and the fraction of protein associated with milk fat globule membranes (MFGMs). Proteins in skimmed milk decreased significantly after treatment in four samples analyzed. Looking at the whole cohort, this decrease in protein appears to be significant, which is in agreement with the findings of Vieira and colleagues[27]. Moreover, the analysis of the skimmed phase of the milk at lower molecular weight, using the MALDI-TOF profiling, shows no structural or qualitative change in the proteins: the only change in skimmed milk is, therefore, quantitative. On the other hand, the proteins associated with fat globule membranes appear to be increased after pasteurization. This opposite protein concentration variation observed in our study between skimmed milk and MFGM may be due to different concomitant factors. First, a reduction in protein content caused by the denaturation of some proteins belonging to immunoglobulins, enzymes, cytokines, and growth factors as indicated in[19] caused by Holder pasteurization treatment. Then, an interaction and subsequent aggregation between proteins present in the skimmed milk phase and those associated with MFGMs, as proposed by Ma and colleagues[28]. The presence of more intense bands in SDS-PAGE profiles of pasteurized MFGM could be attributed to the additional amount of lactoferrin and β casein moved from the aqueous milk phase during pasteurization[29]. Otherwise, the fat globule membrane may reorganize during pasteurization. This change most likely involves an increase in the volume of the fat globule, which is achieved by a physical transfer from the skimmed milk fraction. This hypothesis aligns with recent findings observed on the effects of various thermal treatments of cow's milk fat globules[30].

Our study's original contribution is to investigate the presence of the eNAMPT/visfatin in skimmed milk and MFGM-associated proteins and its potential quantitative change after the Holder pasteurization process. Indeed, although the presence of eNAMPT/visfatin has already been described in skimmed milk[9], to our current

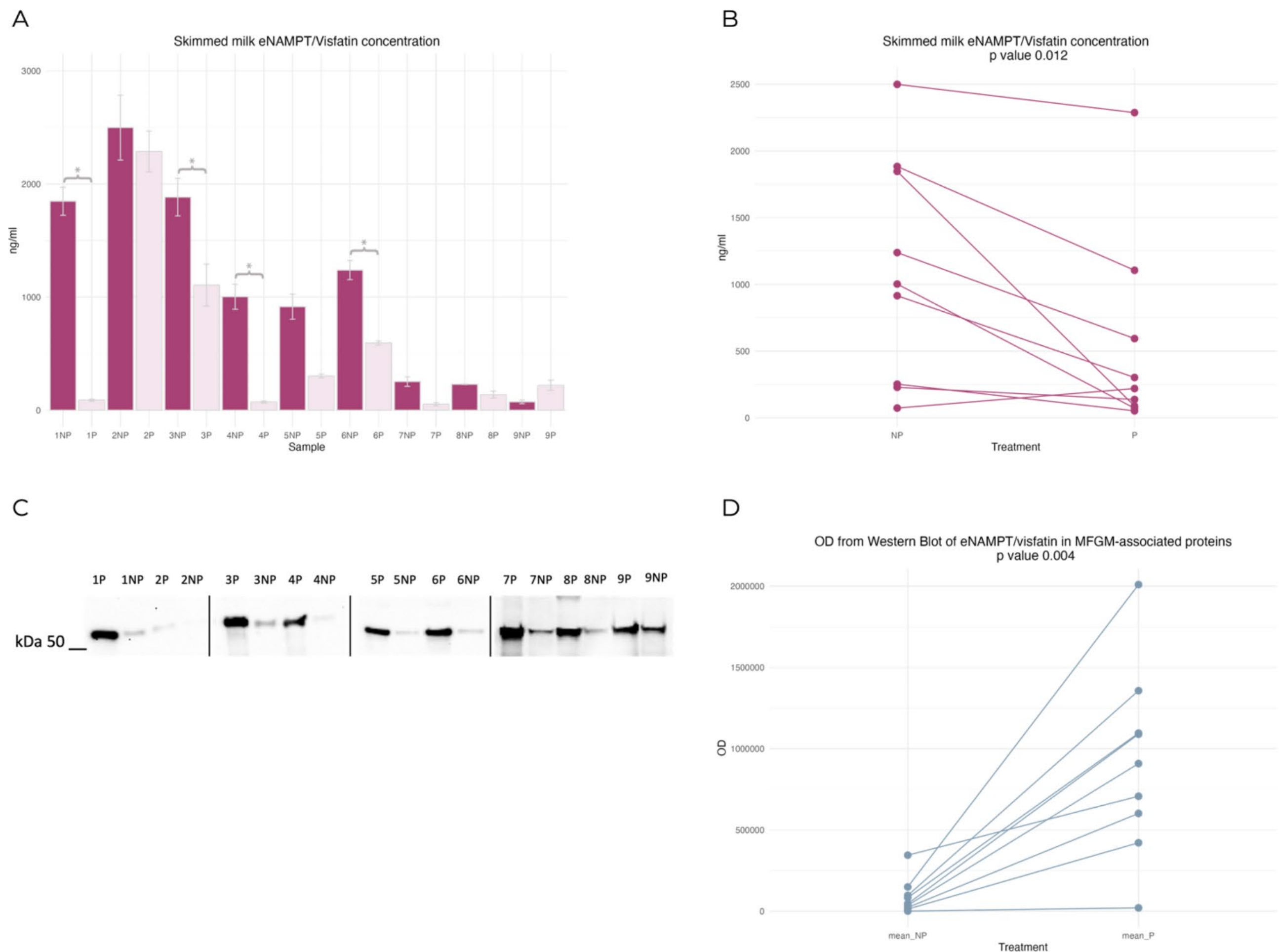

Fig. 2. Analysis of eNAMPT/Visfatin of not pasteurized (NP) and pasteurized samples (P). Panel (**A**) shows the concentration of eNAMPT/Visfatin in skimmed milk obtained by ELISA assay. Data are expressed in ng/ml and presented as mean visfatin concentration, derived from two technical replicates ± standard deviation. In addition, statistical significance, calculated by t-test, is indicated by "***" (p-value < 0.001), "**" (p-value < 0.01), and "*" (p-value < 0.05). Panel (**B**) reports the concentration of eNAMPT/Visfatin expressed in ng/ml. Panel (**C**) shows the immunoreactive bands in P and NP samples. Panel D reports the optical density analysis of the Western Blot present in (**C**). (**B** and **D**) represent the data studied as a cohort; statistical analysis was performed with the Wilcoxon matched-pairs signed rank test.

knowledge, the presence of eNAMPT associated to milk fat globules has not been reported in the literature before. Furthermore, the effect of pasteurization on this protein is unknown. The search for any differences between raw and subsequently pasteurized samples was carried out using two methodologies according to the nature of the samples. The skimmed milk phase, i.e., an aqueous phase, was investigated by a specific ELISA assay. MFGM-associated proteins, on the other hand, were analyzed by Western Blot because the extraction and solubilization buffer for these is highly denaturing and, when used in ELISA assay, caused denaturation of the antibody-protein sandwich. The ELISA results demonstrate decreased eNAMPT/visfatin concentration in samples subjected to Holder pasteurization with a loss of nearly 50% of eNAMPT/visfatin amount after treatment. This could be explained by a possible denaturation of the protein or by its increased affinity to the fat phase (and thus easier transferring to the MFG surface). The western blot of MFGM-associated proteins also confirms the trend. Here, very intense immunoreactive bands can be observed in the pasteurized samples and less intense bands in the raw samples, suggesting the transfer of the eNAMPT/visfatin protein within or in association with the MFGMs. Indeed, this increase is confirmed by statistical analysis of their optical density (OD) value, which allows us to see a 10-fold increase in the quantity of eNAMPT/visfatin in the samples after pasteurization.

For the first time, the presence of eNAMPT/visfatin was demonstrated not only in the skimmed milk phase, already reported in the literature, but also in the protein phase associated with milk fat globule membranes. Holder pasteurization also affects this protein: its concentration decreases in the skimmed phase of pasteurized milk and increases in the protein-associated phase of MFGM. This can be explained by assuming that this protein was plausibly associated with a higher affinity for fat globules during their reorganization caused by the heat treatment. The eNAMPT/visfatin association with MFGM could support the hypothesis of its involvement in fat metabolism and infant body weight regulation[9,11]. We can further suggest a potential active role of eNAMPT/

visfatin in the immunological *status* of newborns[31]. Further investigation of MFG entrapment of eNAMPT/visfatin, which is a soluble protein, could add information on the role of this released cytokine.

Study limitation. The study presented in this manuscript did not investigate the thermal resistance of eNAMPT/visfatin. Reduced thermal resistance resulting in denaturation could contribute to the decreased concentration of eNAMPT/visfatin in pasteurized milk. In addition, the association of soluble visfatin with milk fat globules during pasteurization has not been investigated directly. Since milk is an unstable emulsion composed of aqueous and oil phases, we cannot assess with certainty that the preparations tested in Western blot contained only milk fat globules, they corresponded to the proteins extracted from the floating milk fat layer.

Methods

Human milk samples

In this study, we investigated human milk samples kindly provided by DHMB of Ospedale Sant'Andrea, Vercelli, Italy. The mothers gave informed and signed consent to the study. This project was approved by the Department of Science and Technological Innovation of the University of Piemonte Orientale (PROT #0006564–23/12/2021). All the experiments were performed in accordance with relevant guidelines and regulations. In particular, the recommendations stated in[15] were followed for donated human milk samples. Between January and December 2023, 10 milk samples were collected from 10 healthy lactating mothers. Donor exclusion criteria are reported in[15]. DHMs were subjected to bacteriological controls to exclude samples with theoretical risk for bacteria > 10^5 CFU/ml. For DHM contaminated by *Staphylococcus aureus* and Enterobacteriaceae, discharge was warranted if the contamination exceeded 10^4 CFU/ml. Immediately after donation, an aliquot was separated from the bottle and frozen at -20 °C. Another aliquot was pasteurized for 30 min at 62,5°C (Holder pasteurization process) and then frozen at -20 °C. The samples were transported, guarantying the cold chain, to the biochemistry laboratory and were stored at -80 °C until analysis.

Milk phase separation and protein quantification

A protease inhibitor cocktail (cOmplete, Mini; Roche) was added to the milk aliquots, and the samples were then centrifugated at 2,000 x g for 10 min at 10 °C to allow the separation of skimmed milk from fat globules (MFG). MFGs were washed with 0.9% saline and centrifuged at 3,000 x g for 10 min at 10 °C three times, as indicated in[32]. Proteins associated with MFG membranes (MFGM) were extracted with an estimated equal volume of 2D buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 100 mM DTT, IPG-buffer (pH 3–10)). The protein concentration of both phases, skimmed milk and MFGM, was quantified using an optimized Bradford microplate assay with BSA (bovine serum albumin) as standard[33].

Protein profiling

Samples were analyzed by SDS-PAGE. Proteins (10 µg) were mixed to an equal volume of Laemmli reducing sample buffer[34] and heated at 95 °C for 5 min. Skimmed milk proteins were loaded onto 12.5% polyacrylamide gel, and MFGM-associated proteins were loaded onto 10% polyacrylamide gel. SDS-PAGE was performed at 100–120 V with a Mini Protean System (BioRad) using a molecular weight protein standard (Precision Plus Protein ™ Dual Color Standard, BioRad). Gel staining was carried out with Blue Silver Stain[35]. After destaining, the gel images were obtained with a GS-900 densitometer (BioRad).

Skimmed milk proteins were also analyzed through the MALDI-TOF spectrometer. One µl of samples at 1 mg/ml protein concentration was mixed with 10 µl of matrix solution (10 mg/ml sinapinic acid dissolved in acetonitrile: 0.1% TFA, 30:70% v/v – TA30), and 1 µl of each mix was spotted on a ground steel MTP 384 target plate (Bruker Daltonics GmbH, Bremen, Germany) in triplicate and let dry at room temperature overnight. MALDI-TOF spectra were acquired by Ultraflextreme mass spectrometer (Bruker Daltonics GmbH, Bremen) equipped with a Smart-beam 2 Nd: YAG laser operating at 355 nm, using FlexControl software (version 3.3, Bruker Daltonics GmbH, Bremen, Germany). Skimmed milk proteins were analyzed in linear mode in positive ion acquisition in a mass-to-charge ratio range of 2,000–50,000 and reflectron mode in positive ion acquisition in a mass-to-charge ratio of 700–3,500. The mass spectra obtained are the sum of 6000 shots for each sample, and the random walk feature with partial sample mode was activated. Protein mass spectra were calibrated through "Protein Calibration Standard I", hydrolysates using "Peptide Calibration Standard I" (Bruker Daltonics GmbH, Bremen) in FlexAnalysis software (version 3.3, Bruker Daltonics GmbH, Bremen).

To analyze the principal component of spectra these were subjected to GeenaR (https://proteomics.hsanmartino.it/geenar/) with the following parameters: trimming from 1,400 to 3,500 (for spectra analyzed with reflectron method) or 2,000 to 30,000 (for spectra analyzed with the linear method); variance stabilization: square root; smoothing: Savitzky–Golay with half window size of 10; baseline removal: TopHat, with half window size of 75; normalization: total ion current (TIC). Peak alignment was carried out using MAD (median absolute deviation) for noise estimation (half window size: 20, SNR: 2) and LOWESS (local weight scatterplot smoothing) for phase correction (mass tolerance: 0.002). Then, peaks were selected by applying the strict method for binning and a coverage (minimum frequency of peaks for their selection) of 0.5. Finally, principal component analysis was applied to the processed mass spectra[36].

eNAMPT/visfatin detection

A western blot was performed to detect the presence of eNAMPT/visfatin (UniProt P43490) in MFGM proteins. 20 µg of proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4–20% Cat. #4568093) and transferred using Trans-Blot® TurboTM Transfer System to nitrocellulose membranes (all from Bio-Rad). A recombinant NAMPT (rec) form was prepared in-house[37] and used as a control. To detect NAMPT, an anti-NAMPT was used (A300-779 A, Bethyl Laboratories, Montgomery, TX, USA) with a mouse anti-rabbit HRP-conjugated as the secondary antibody (sc-2357, Santa Cruz Biotechnology). Western blot

chemiluminescence reactions were visualized with ECL (Cat. #170–5061) (Bio-Rad, Segrate, Milan, IT) using the Invitrogen iBright CL1500 Imaging System (Thermo Scientific). Densitometric analyses were performed using Image Lab 6.1 Software (Bio-Rad) or iBright Analysis Software 5.2.1 (Thermo Scientific).

Skimmed milk eNAMPT/visfatin concentrations in not pasteurized and pasteurized milk were determined using a human NAMPT Enzyme-Linked Immunosorbent Assay (ELISA) kit (Adipogen, AG-45 A-0006YEK-KI01). Samples were diluted 300-fold to the assay range (0.125-8 ng/ml) with assay diluent, and the assay was performed according to the manufacturer's instructions[9].

Statistics

The pairwise t-test was used to compare continuous variables, while the nonparametric pairwise t-test was used to analyze cohort data. Unless otherwise indicated, data are represented as mean ± SD. Statistical analyses were performed on RStudio version 2023.12.0 + 369. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Data availability

The sequence of eNAMPT/visfatin analyzed during the current study is available in the UniProt repository (UniProt P43490). The human milk protein sequences cited in the discussion section are available in the UniProt repository with the following accession numbers: lactoferrin (P02788), serum albumin (P02768), immunoglobulin heavy chain (P01877), and beta-casein (P05814).

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Author contributions
A.G, V.A., and M.C. devised the manuscript; A.G., C.G., I.F., and A.M.T. studied the samples; C.G. M.B., E.U., and G.C. collected the samples; A.G, V.A., and M.C wrote and revised the manuscript; V.A. and M.C supervised the whole research. All authors revised the manuscript.

Declarations

Competing interests
The authors declare no competing interests.

Additional information
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