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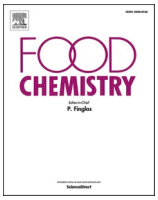

Beyond the gut: Investigating the mechanism of formation of β-casomorphins in human blood

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ABSTRACT

To evaluate the potential differences in the propensity of β-casein A1 (β-CNA1) and A2 (β-CNA2) from bovine milk to release health-relevant β-casomorphins (BCMs), food-derived peptides were monitored over time in the blood of eight human volunteers who consumed milk containing both protein variants. Liquid chromatography coupled with high resolution tandem mass spectrometry revealed interindividual variability of milk peptidomic profiles in human blood. BCMs were not detected, whereas BCM precursors originating from both β-CNA1 and β-CNA2 were ascertained, with β-CNA2-derived peptides showing a slightly greater susceptibility to proteolysis. Ten synthetic peptides mimicking circulating BCM precursors from β-CNA1 and β-CNA2, which were incubated *ex vivo* with the blood of two volunteers, showed comparable potential to generate BCMs. The formation of BCMs seemed to depend mainly on the size of the BCM precursors and less on the presence of His^{67} or Pro^{67}. These findings challenge the belief that BCMs are released exclusively from β-CNA1 and support the nutritional safety of conventional milk, informing health policies regarding milk consumption.

1. Introduction

The debate on β-casomorphins (BCMs) has spanned decades, evolving from recognition as beneficial peptides with opioid-like agonist properties to associations with various metabolic disorders, earning the nickname "the devil in the milk" (Borş, Borş, & Florișteanu, 2024; Küllenberg de Gaudry et al., 2019; Ul Haq, Kapila, Shandilya, & Kapila, 2014; Woodford, 2009). Constituting approximately 30% of bovine milk proteins, β-casein (β-CN) exists predominantly in the genetic variants A1 (β-CNA1) and A2 (β-CNA2), which differ by the $His^{67} \rightarrow Pro^{67}$ substitution. Epidemiological studies have associated the consumption of β-CNA1-containing milk with various adverse health effects, including increased risks of childhood type 1 diabetes, coronary heart disease, and autism (Elliott, 1992; Elliott, Harris, Hill, Bibby, & Wasmuth, 1999; Laugesen & Elliott, 2003; McLachlan, 2001). To link structural data with biological activity, it was suggested that His^{67} in β-CNA1 promotes a more efficient release of β-casomorphin-7 (BCM7) during gastrointestinal digestion, whereas Pro^{67} in β-CNA2 hampers peptide formation (Jinsmaa & Yoshikawa, 1999). Later, Truswell argued against any link between β-CNA1 intake and diabetes or cardiovascular diseases (Truswell, 2005), whereas Kamiński and colleagues reinforced the pathological causative relationship on the basis of data regarding *in vitro* gastrointestinal digestion of β-CN (Kamiński, Cieslińska, & Kostyra, 2007). In 2009, a panel of European Food Safety Agency experts excluded any deterministic connection between milk consumption, BCMs uptake and adverse health effects (EFSA Scientific Report, 2009).

In subsequent studies, BCMs were identified after *in vitro* simulated gastroduodenal-intestinal digestion of milk (Picariello et al., 2015), as

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well as in human jejunal aspirates following milk ingestion (Boutrou et al., 2013). The detection of BCM7 at comparable levels after *in vitro* and *ex vivo* digestion of β-CNA1 and β-CNA2 revealed that the release of BCM7 is not exclusive to β-CNA1 (Asledottir et al., 2017; Asledottir, Poulsen, Devold, Larsen, & Vegarud, 2018). Recent investigations have confirmed the generation of BCM7 and longer BCM precursors during *in vitro* gastroduodenal digestion (Lambers, Broeren, Heck, Bragt, & Huppertz, 2021; Reiche et al., 2024), whereas slightly different tendencies of β-CNA1 and β-CNA2 to release BCM7 have been ascribed to fluctuations in the activity of digestive enzymes (Cattaneo, Masotti, Stuknytė, & De Noni, 2023).

Hypotheses about the mechanisms through which BCM7 might exert harmful effects have intensified the debate (Trivedi, Zhang, Lopez-Toledano, Clarke, & Deth, 2016; Woodford, 2021). BCM7 can delay intestinal motility by agonizing local μ-opioid receptors, thus interfering with the gut-brain axis (Summer, Di Frangia, Ajmone Marsan, De Noni, & Malacarne, 2020). The pathological multi-organ effects listed above were associated with systemic, chronic exposure to exogenous opioid peptides (Thiruvengadam, Venkidasamy, Thirupathi, Chung, & Subramanian, 2021; Woodford, 2021). However, the effects of BCMs on the central nervous system were demonstrated after intraperitoneal/intracerebral administration in mice, under conditions different from those of dietary intake (Dubynin et al., 2008; Lin, Umahara, York, & Bray, 1998). To exert any influence on the human central nervous system, BCMs must cross the intestine-blood and blood-brain barriers and should have sufficient molecular stability and efficient distribution in these anatomical regions. Therefore, some authors have suggested that BCMs might exert harmful effects only in individuals with impaired functionality of anatomical barriers, such as those with increased intestinal permeability (Daniloski, McCarthy, & Vasiljevic, 2021; Jeong, Park, & Yoon, 2024). Recent research has detected BCM7 among milk-derived bioactive peptides in human blood, suggesting potential direct systemic effects (Tagliamonte et al., 2023). However, evidence concerning the formation, absorption, and stability of BCM7 in human blood is controversial because of its rapid hydrolysis by plasma peptidases (Caira et al., 2022a; De Pascale et al., 2024). Similarly, the evolution of β-CNA1- and β-CNA2-derived peptides in human blood remains unknown. These knowledge gaps underscore the need to unravel the mysteries surrounding BCM7 and its potential health implications (de Vasconcelos, Oliveira, Hill, & Vidal, 2023).

Assessing the presence of bioactive peptides in human blood is key to determining their potential systemic activity (Caira et al., 2022a). In this study, nanoflow-liquid chromatography coupled with high-resolution tandem mass spectrometry was used to monitor BCMs and other milk protein-derived peptides in the bloodstream of eight human volunteers who had consumed milk with defined levels of β-CNA1 and β-CNA2 variants. The dominant circulating peptides encrypting the BCM7 sequence were subsequently synthesized and individually incubated *ex vivo* with human blood to trace their evolution. This study sought to provide novel insights into the post-ingestion kinetics of milk-derived peptides in healthy adults, with a special emphasis on BCMs.

2. Material and methods

2.1. Milk, chemicals and reagents

Commercial UHT bovine milk (a 5 L batch from the same company and with the same expiration date) was purchased from a local retailer at a supermarket (Naples, Italy). Aliquots from this milk batch were used for the intervention study and were administered to all the volunteers.

Synthetic peptides, synthesized using solid-phase with Fmoc chemistry, were purchased from Bio-Fab Research (Rome, Italy). LC-MS grade acetonitrile, methanol, water, and formic acid were obtained from Merck-Sigma (Darmstadt, Germany). Other reagents were of analytical grade and were purchased from Merck-Sigma.

2.2. Determination of the contents of β-CNA1 and β-CNA2 variants in UHT bovine milk

To determine the relative contents of the β-CNA1 and β-CNA2 variants, a 10 mL sample from the above-reported UHT milk batch was defatted by removing the fat layer resulting from centrifugation (5000*g*, 30 min, 4 °C). The skim milk was diluted 1:1 (*v*/v) with deionized water and the pH value of the resulting mixture was adjusted to 4.6 with 10% (*v*/v) acetic acid. After casein (CN) precipitation, the milk was centrifuged (5000*g*, 20 min, 4 °C), and the supernatant was discarded. The pellet was resuspended in 40 mL of 0.1 mM sodium acetate, pH 8.8, and CN was precipitated at pH 4.6 with 10% (v/v) acetic acid. The solution was centrifuged again, and the supernatant was discarded. The pellet was resuspended in 0.1 mM sodium acetate at a 2 mg/mL concentration, and an aliquot (0.1 mg) was separated by high-performance liquid chromatography (HPLC) (Visser, Slangen, & Rollema, 1991) using an HP 1100 chromatographer (Agilent Technologies, Paolo Alto, CA, USA) equipped with a multi-wave UV detector. For this purpose, a reversed phase (RP) C_4 Jupiter® column (250 × 2.1 mm, 5 μm particle diameter, 300 Å pore size, Phenomenex, Torrance, CA, USA) was used, which was eluted with 0.1% (v/v) trifluoroacetic acid (TFA) in water (solvent A) and 0.1% (v/v) TFA in acetonitrile (solvent B). Casein was separated by applying a 30–50% B linear gradient over 50 min, at a flow rate of 0.2 mL/min. The eluate was monitored at 214 and 280 nm. Analysis was performed in technical triplicate. Chromatographic peaks were integrated and elaborated with the ChemStation software version A.07.01 (Agilent Technologies), demonstrating the occurrence of β-CNA1 and β-CNA2 variants at proportions of 36% and 64%, respectively (**Supplementary Fig. S1**).

2.3. Study participants and blood sample collection

The study was conducted on eight healthy volunteers (3 males and 5 females) aged between 30 and 50 years, with body weights ranging from 55 to 72 kg. The participants provided written informed consent prior to enrollment, and the study adhered to the principles of the Declaration of Helsinki. Ethical approval was obtained from the Ethics Committee of the University of Naples Federico II (protocol number 266/17). All the volunteers adhered to a washing-out period consisting of a milk- and dairy-free diet one week before nutritional intervention. On the sampling day, after overnight fasting, participants were cannulated at the brachial vein, and blood samples were obtained at baseline ($t = 0$) from each volunteer. Immediately after, the volunteers were asked to drink 250 mL of UHT bovine milk, and blood samples were collected at 0.5 h ($t = 1$), 1 h ($t = 2$), 2 h ($t = 3$), 3 h ($t = 4$), 4 h ($t = 5$), 5 h ($t = 6$), 6 h ($t = 7$), 7 h ($t = 8$), and 8 h ($t = 9$) after milk consumption. Then, the volunteers were allowed to consume a dairy-free meal and underwent a final blood sampling after 24 h ($t = 10$). Overall, 11 blood samples were obtained from each volunteer. Among the participants, one individual (volunteer #1) had abstained from consuming milk or cheese for one year, whereas the remaining individuals were regular dairy product consumers. Blood samples were collected using an established protocol (Caira et al., 2022b). Specifically, blood was collected in 3 mL vacutainer tubes containing EDTA and immediately centrifuged at 4,000*g* for 10 min, at 4 °C, to deplete the corpuscular fraction. The resulting plasma samples were aliquoted within 20 min of collection and stored at −80 °C until further use. We considered the potential impact of freezing on fatty structures in plasma, which may alter the concentration of milk-derived peptides by making them more accessible in the nonfatty part of the samples. However, this aspect was not further examined in the present study.

2.4. Purification of milk-derived peptides from human blood

Consistent with a previous study (Caira et al., 2022b), frozen plasma samples (1 mL each) were rapidly thawed in an ice-cold bath and then

diluted tenfold with a denaturing buffer composed of 6 M guanidine, 50 mM Tris, and 1 mM EDTA, pH 8.0. The polypeptide components in 1 mL of each mixture were subsequently fractionated using size-exclusion chromatography prepacked columns with an exclusion limit of 6 kDa (Econo-Pac 10 DG, Bio-Rad, Hercules, CA 94547, USA), which were eluted with 50 mM ammonium bicarbonate, pH 7.8. Fractions (1 mL each) were manually collected and monitored by UV at 280 nm using an Ultrospec 2100 UV–Visible spectrophotometer (GE-Healthcare, Uppsala, Sweden). Fractions containing peptides were pooled and desalted using C_{18} prepacked Sep-Pak cartridges (Waters-Millipore, Bedford, MA, USA), which were subsequently washed with 0.1% (*v*/v) TFA and finally eluted with 60% (v/v) acetonitrile/0.1% (v/v) TFA. Peptide quantification was performed using a modified micro-Lowry assay (Sigma-Aldrich). Finally, the pooled eluates of individual blood samples were vacuum concentrated and lyophilized, and the peptides were reconstituted in 0.1% (v/v) formic acid.

2.5. Untargeted LC-ESI-Q-Orbitrap-MS/MS analysis

NanoLC-ESI-Q-Orbitrap-MS/MS analyses were carried out using an Ultimate 3000 ultrahigh-performance liquid chromatography instrument (Thermo Fisher Scientific, San Jose, CA, USA) coupled to a Q Exactive Orbitrap Plus mass spectrometer (Thermo Fisher Scientific). For each peptide pool, 2 μg was loaded using the autosampler onto an RP C_{18} pre-column (5 mm, 300 μm i.d.) (LC Packings, USA) linked to an EASY-Spray™ PepMap RP C_{18} column (15 cm × 75 μm, 3 μm particle diameter, 100 Å pore size) (Thermo Fisher Scientific). Eluent A was 0.1% (v/v) formic acid in water; eluent B was 0.1% (v/v) formic acid in acetonitrile. The column was equilibrated at 3% B. Peptides were separated by applying a 3–45% gradient of eluent B for 60 min, at a flow rate of 300 nL/min. The mass spectrometer operated in data-dependent mode, and all MS1 spectra were acquired in positive ionization mode, scanning the range *m/z* 350–1600. The top 10 ions in MS were selected for fragmentation in MS/MS mode. Precursor spectra were generated at a 70,000 full width at half maximum (FWHM) resolving power, with automatic gain control (AGC) targets of 1×10^6 and 1×10^5 ions for full MS and MS/MS spectra, respectively; a maximum ion injection time of 100 ms was used. MS/MS fragmentation spectra were obtained at a resolving power of 17,500 FWHM, with a dynamic exclusion of 10 s. Parent ions with a net charge greater than 6 were excluded. Data analysis was performed using Xcalibur software (version 3.1, Thermo Fisher Scientific). Measurements were performed on blood samples from eight volunteers, per eleven time points, in technical triplicate, resulting in 264 independent observations.

2.6. Peptide identification and computational analysis

NanoLC-ESI-Q-Orbitrap-MS/MS raw files resulting from three technical replicates of each plasma sample were uploaded into the Andromeda search engine of the MaxQuant bioinformatic suite (version 1.6.2.10). The technical replicates were identified within Andromeda to ensure proper handling as replicates during analysis. Peptide quantification was conducted using the Andromeda's label-free quantification (LFQ) option. Raw data searches were taxonomically restricted to *Bos taurus* in the UniProtKB database (downloaded in December 2020); subsequently, they were refined using a manually constructed protein database containing the 30 most abundant bovine milk gene products inferred from proteomic-based investigations (Picariello et al., 2019). Additional searches were performed to identify endogenous peptides, extending the taxonomy to the *Homo sapiens* UniProtKB protein database (also downloaded in December 2020). For all raw data searches, the following parameters were used: mass tolerance values of 10 ppm for the precursor and 0.02 for the fragment ions; no proteolytic cleavage specificity; and Met oxidation, pyroglutamic acid formation at the N-terminal Gln, and Ser/Thr phosphorylation as variable modifications. Peptide spectrum matches (PSMs) were filtered using the target decoy database approach with an e-value of 0.01 peptide-level false discovery rate (FDR), corresponding to a 99% confidence score. To ensure robustness and confidence in the analysis, the output peptide.txt file was filtered to include data for peptides simultaneously and confidently identified in all three replicates at a given time point, including corresponding PSMs. The measured ion intensities (calculated as ion counts) by Andromeda were averaged for each peptide across all replicates. Inter-replicate reproducibility was assessed using Pearson correlation at both the peptide and protein levels with the Perseus suite of MaxQuant software (version 1.6.15.0).

2.7. Visualization of peptidomic data

Peptide maps were visualized using the Peptigram web application (http://bioware.ucd.ie/peptigram/). Relative peptide abundances were inferred from the MS signal ion count. In the resulting graphs, peptide entries and their corresponding abundances, represented with different shades of green, were aligned to the parent protein sequence.

2.8. Synthetic BCM precursors

Ten BCM precursors were synthesized based on the sequence of peptides containing the β-casomorphin region found in the blood samples (see below). As stated above, the peptides were provided by Bio-Fab Research (Rome, Italy); peptide identity and purity were confirmed by MALDI-TOF-MS and/or MALDI-TOF-TOF-MS analysis using an UltrafleXtreme MALDI-TOF-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) and α-cyano-4-hydroxycinnamic acid (4 g/L) in 60% (*v*/v) aqueous acetonitrile containing 0.1% v/v TFA as the matrix (Loebel et al., 2000). Synthetic peptides were fully characterized according to their spectral profile, experimental mass value and fragmentation data. They were used as substrates for *ex vivo* proteolysis experiments with human blood samples and were divided into two groups according to the presence of His or Pro at position 67.

2.9. Collection of blood samples for ex vivo proteolysis experiments on synthetic peptides

Blood samples for *ex vivo* proteolysis experiments were obtained from two healthy human individuals different from those reported above, *i.e.*, volunteers #9 and #10, a 60-year-old male and a 55-year-old female. Before blood sampling, volunteers provided informed consent and refrained from consuming milk and dairy products for at least one week. No additional dietary restrictions were imposed. Blood was drawn by a skilled operator from the volunteers' brachial veins following a 12-h fasting period and collected into multiple 3 mL vacutainer tubes containing EDTA. The collected blood samples were gently mixed through tube inversion and immediately used for the subsequent BCM precursor spiking procedures.

2.10. Ex vivo incubation of synthetic BCM precursors with blood and purification of proteolytic products

In general, studies of polypeptide proteolytic stability in human blood are carried out under non-standardized conditions (Cavaco, Andreu, & Castanho, 2021). The ratio between blood enzymes and peptide substrate was chosen based on previous experimental findings (De Pascale et al., 2024); this ratio represents a compromise between the putative concentration of food-derived molecules in blood and the optimal detectability of synthetic peptide proteolytic products. Briefly, 200 μL of EDTA-treated blood sample from each participant was mixed with 200 μL of an aqueous solution of each synthetic peptide (6 nmol/mL). The experiments were conducted in technical duplicate in sterile glass tubes. The final concentration of each synthetic peptide roughly corresponded to the levels of compounds previously used for *ex vivo* monitoring of peptides in human plasma (Bottger, Hoffmann, & Knappe,

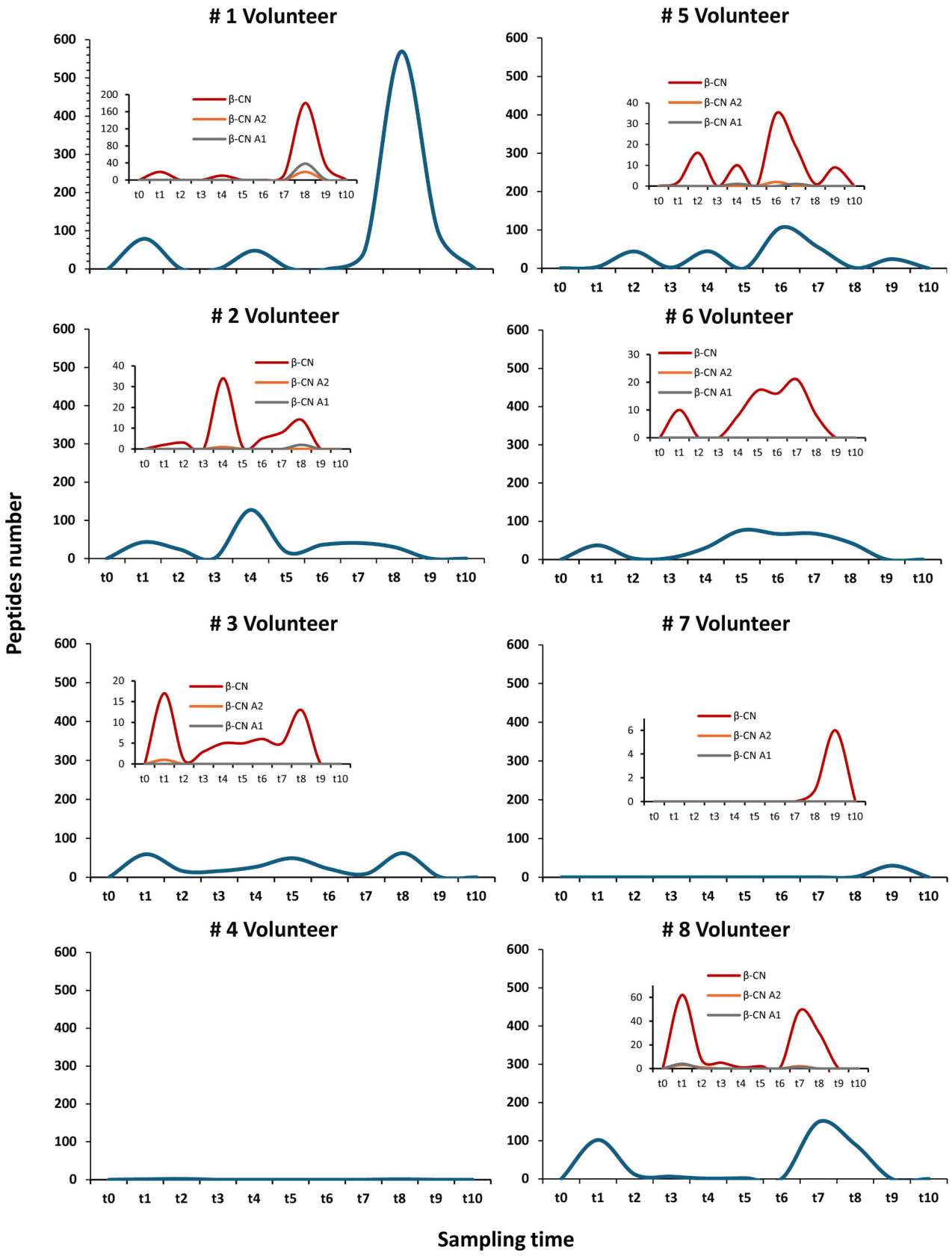

Fig. 1. Time-course distribution of milk-derived peptides detected in the blood of eight volunteers administered 250 mL of UHT bovine milk. The inset shows the distribution of the detected β-CN-derived peptides.

Table 1
Comparative analysis of *in vivo* representation at different times of CN-derived peptides in the blood of eight volunteers administered 250 mL of UHT bovine milk. The total number of detected peptides and their distribution within the CN fraction are reported. A significant level of variability among the volunteers was observed. The peptide counts for β-CN A1 and A2 specify only the number of peptide sequences, including His[67] and Pro[67], respectively.

Volunteer		Withdrawal number	t0	t1	t2	t3	t4	t5	t6	t7	t8	t9	t10
		Time (min/h)	t=0	t=30 min	t=1h	t=2h	t=3h	t=4h	t=5h	t=6h	t=7h	t=8h	t=24h
#1	CN fraction	β-CN	0	19	0	0	10	0	0	13	180	34	0
		β-CN A2	0	0	0	0	0	0	0	0	19	0	0
		β-CN A1	0	0	0	0	0	0	0	0	38	1	0
		αs1-CN	0	11	0	0	4	0	0	5	175	21	0
		αs2-CN	0	1	0	0	0	0	0	2	58	7	0
		κ-CN	0	13	0	0	13	0	0	6	56	12	0
		Total CN peptides	0	44	0	0	27	0	0	26	526	75	0
#2	CN fraction	β-CN	0	2	3	0	34	1	5	8	14	0	0
		β-CN A2	0	0	0	0	1	0	0	0	0	0	0
		β-CN A1	0	0	0	0	0	0	0	0	2	0	0
		αs1-CN	0	2	3	0	10	0	3	1	5	0	0
		αs2-CN	0	0	0	0	0	0	0	2	1	0	0
		κ-CN	0	7	7	0	15	3	10	8	4	0	0
		Total CN peptides	0	11	13	0	60	4	18	19	26	0	0
#3	CN fraction	β-CN	0	17	1	3	5	5	6	5	13	0	0
		β-CN A2	0	1	0	0	0	0	0	0	0	0	0
		β-CN A1	0	0	0	0	0	0	0	0	0	0	0
		αs1-CN	0	5	0	0	1	2	1	1	10	0	0
		αs2-CN	0	0	0	1	1	1	0	0	3	0	0
		κ-CN	0	5	4	1	4	9	1	0	8	0	0
		Total CN peptides	0	28	5	5	11	17	8	6	34	0	0
#4	CN fraction	β-CN	0	0	0	0	0	0	0	0	0	0	0
		β-CN A2	0	0	0	0	0	0	0	0	0	0	0
		β-CN A1	0	0	0	0	0	0	0	0	0	0	0
		αs1-CN	0	1	2	0	0	0	0	0	0	0	0
		αs2-CN	0	0	0	0	0	0	0	0	0	0	0
		κ-CN	0	0	0	0	0	0	0	0	0	0	0
		Total CN peptides	0	1	2	0	0	0	0	0	0	0	0
#5	CN fraction	β-CN	0	2	16	0	10	0	35	19	1	9	0
		β-CN A2	0	0	0	0	0	0	2	0	0	0	0
		β-CN A1	0	0	0	0	1	0	0	1	0	0	0
		αs1-CN	0	1	14	0	14	0	39	20	0	5	0
		αs2-CN	0	1	11	2	16	0	20	12	0	8	0
		κ-CN	0	0	1	0	3	0	8	2	0	1	0
		Total CN peptides	0	4	42	2	44	0	104	54	1	23	0
#6	CN fraction	β-CN	0	10	0	0	8	17	16	21	8	0	0
		β-CN A2	0	0	0	0	0	0	0	0	0	0	0
		β-CN A1	0	0	0	0	0	0	0	0	0	0	0
		αs1-CN	0	13	1	1	15	33	26	21	18	0	0
		αs2-CN	0	10	0	2	6	20	17	17	11	0	0
		κ-CN	0	3	0	0	2	4	7	4	6	0	0
		Total CN peptides	0	36	1	3	31	74	66	63	43	0	0
#7	CN fraction	β-CN	0	0	0	0	0	0	0	0	1	6	0
		β-CN A2	0	0	0	0	0	0	0	0	0	0	0
		β-CN A1	0	0	0	0	0	0	0	0	0	0	0
		αs1-CN	0	0	0	0	0	0	0	0	0	10	0
		αs2-CN	0	0	0	0	0	0	0	0	0	11	0
		κ-CN	0	0	0	0	0	0	0	0	0	2	0
		Total CN peptides	0	0	0	0	0	0	0	0	1	29	0
#8	CN fraction	β-CN	0	62	7	5	1	2	1	49	30	0	0
		β-CN A2	0	3	1	0	0	0	0	2	0	0	0
		β-CN A1	0	4	0	0	0	0	0	1	0	0	0
		αs1-CN	0	29	3	1	0	0	0	49	29	0	0
		αs2-CN	0	3	1	0	0	0	0	31	19	0	0
		κ-CN	0	1	0	0	0	0	0	7	5	0	0
		Total CN peptides	0	102	12	6	1	2	1	139	83	0	0

2017; Padberg, Schumm-Draeger, Petzoldt, Becker, & Federlin, 1999; Sokolov et al., 2014; Wasilewska et al., 2011). This amount was equivalent to the intake of approximately 1.0 g of β-CN, roughly corresponding to a 200 mL serving of milk, assuming the generation of equal molar amounts of each BCM precursor from the parent protein and the subsequent complete absorption of the peptide into the bloodstream, allowing the optimal detection of the resulting fragments and increasing the physiological relevance of the data (De Pascale et al., 2024). Blood samples without BCM precursors or those inactivated for peptidases were prepared as control samples (De Pascale et al., 2024). In the case of peptidase-inactivated blood, EDTA-treated samples were acidified to a pH value of 2 with formic acid (5 μL), vigorously mixed, and then added to the solution of each BCM precursor. In all the cases, proteolysis was conducted at 37 °C under gentle agitation. Aliquots (50 μL) of the incubation mixtures were sampled at 1, 10, 30, 45, 75 and 120 min after the initial addition of blood to the synthetic peptide. The incubation mixtures and control samples were diluted tenfold with 0.2% (*v*/v) formic acid and thoroughly mixed. All samples were subsequently transferred in parallel to 3 kDa Amicon centrifugal filter units (Merck-Sigma), followed by centrifugation at 12,000 ×*g* for 30 min, at 4 °C. The resulting permeates were vacuum-dried, and reconstituted in 50 μL of 0.1% (v/v) formic acid.

2.11. Targeted characterization of products from synthetic peptide ex vivo proteolysis

The evolution of BCM precursors in blood was monitored by an Ultimate 3000 RS liquid chromatographic system coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA), according to previously detailed conditions (De Pascale et al., 2024). For each blood sample, 5 μL of the reconstituted permeate (0.1% formic acid) was injected, and peptide separation was achieved on an RP C_{18} Luna Omega column (2.6 μm particle size, 100 × 2.1 mm, Phenomenex), which was thermostated at 35 °C and eluted at a flow rate of 0.2 mL/min. The mobile phases were 0.1% (v/v) formic acid in water (solvent A) and 0.1% (v/v) formic acid in methanol (solvent B). The full-scan positive ion acquisition mode was used in the *m/z* range 75–1400. A targeted peptide list was generated for all the theoretical sequences formed upon proteolysis of synthetic peptides (2–12 amino acids). Monoisotopic masses $[M + H]^+$ were extracted by using a mass accuracy ±5 ppm in the Xcalibur 2.1 environment (Thermo Fisher Scientific). Measurements were performed on ten synthetic peptides, each of which parallelly challenged with independent blood samples from two volunteers, per six time points, in technical duplicate, resulting in 240 independent observations.

2.12. PCA multivariate data analysis

Principal component analysis (PCA) included average area counts of each synthetic peptide carrying His^{67} or Pro^{67} ($n = 10$) per 2 volunteers, per six time points, in technical duplicate. The variables were loaded into the Perseus tool of the MaxQuant suite (Max Planck Institute) and log2-transformed. Missing values were replaced with 0 before the analysis (Tyanova, Temu, & Cox, 2016).

3. Results and discussion

3.1. Monitoring milk protein-derived peptides in human blood

Milk protein-derived peptides were monitored in the blood of eight healthy volunteers at various time points within 24 h after they consumed 250 mL of UHT bovine milk. Peptidomic analysis confirmed the absorption of milk protein-derived molecules in the systemic circulation and their transient presence due to proteolysis and clearance. Peptides were detected as early as 0.5 h post-milk ingestion in 7 out of 8 volunteers, exhibiting variable representation patterns over time (**Supplementary Tables S1-S8**, Fig. 1**)**. The highest (677) and the lowest (4) numbers of unique circulating molecules were observed in volunteers #1 and #4, respectively. Volunteers #2, 3, 5, 6 and 8 presented comparable total compound representations over time (191, 153, 150, 121 and 233 unique peptides, respectively). No milk-derived peptides were detected 24 h post-milk ingestion in any of the blood samples.

Fig. 1 shows visual snapshots of the evolution of milk protein-derived peptides in the blood. Individual peptide profiles generally revealed quickly evolving kinetics, as evidenced by the number of protein fragments identified at various time points (Fig. 1 and **Supplementary Tables S1-S8**). Peptide patterns varied significantly among individuals, which is consistent with previous findings (Caira et al., 2022b). This observation underscores the importance of recognizing individual differences in the uptake, degradation, and elimination of milk protein-derived peptides, which could influence the nutritional significance of food proteins. Five out of eight volunteers displayed trimodal distributions of milk peptides in the blood, indicating parallel molecular digestion and absorption patterns. These trimodal peptide distributions might result from a combination of various multifaceted processes, including the functional specificity of the intestinal segments, orocecal transit, enzymatic digestion at the enterocyte and plasma levels, and the intestinal microbiota. Overall, the data vividly revealed the complex topography of the human digestion dynamics of milk proteins.

3.2. Insights into CN-derived peptide absorption kinetics

CN-derived peptides were the most represented molecules in the blood samples, reflecting the abundance of the casein families in milk. The α_{S1}- and β-CN-derived peptides presented the highest representation and intensity, followed by the α_{S2}- and κ-CN-derived peptides (Table 1 and **Supplementary Tables S1-S8**). Individual CN-derived peptide profiles agreed with those reported for the whole set of milk protein-derived profiles (Table 1 and Fig. 1), confirming variations in the molecular representation over time between subjects. Volunteer #1, who consumed dairy products infrequently, exhibited a uniquely high number (526) of circulating CN-derived peptides at 7 h post ingestion (Table 1 and **Supplementary Table S1**), which was compatible with the massive generation of protein fragments at the level of distal intestinal segments, consistent with previous observations (Caira et al., 2022b). Volunteers #2 and #6 presented fewer but significant total circulating CN-derived peptides, peaking at 3–4 h post-ingestion (60–74 molecules) and then declining (Table 1 and **Supplementary Tables S2** and **S6**). Volunteers #4 and #7 presented a minimal number of CN-derived circulating molecules. Specifically, volunteer #4 had only 1–2 peptides at 0.5–1 h post-ingestion, whereas volunteer #7 had no compounds for 7 h, followed by a sudden surge of 29 molecules at 8 h (Table 1 and **Supplementary Tables S4** and **S7**). Volunteers #3, #5 and #8 presented significant but fluctuating influxes of CN-derived peptides peaking at 6–7 h post-ingestion (Table 1 and **Supplementary Tables S3**, **S5,** and **S8**).

3.3. Search for β-casomorphins in human blood

To address concerns related to the potential health effects of β-CNA1, many breeders have undertaken genetic selection programs that have shifted production toward milk containing exclusively β-CNA2 (Fernández-Rico et al., 2022). However, evidence of the exclusive or preferential release of BCM7 during *in vivo* digestion of β-CNA1 and its distribution in the human body remains weak (Asledottir et al., 2018; Lambers et al., 2021; Reiche et al., 2024). To address this issue, we aimed to detect and quantify BCMs, specifically BCM7, in the bloodstream of human volunteers who consumed bovine milk containing defined amounts of β-CN variants; the milk used in this study included β-CNA1 and β-CNA2 at proportions of 36% and 64%, respectively (**Supplementary Fig. S1**). Variable numbers of unique β-CN-derived peptides were detected in the different volunteers: 260 in #1, 52 in #2,

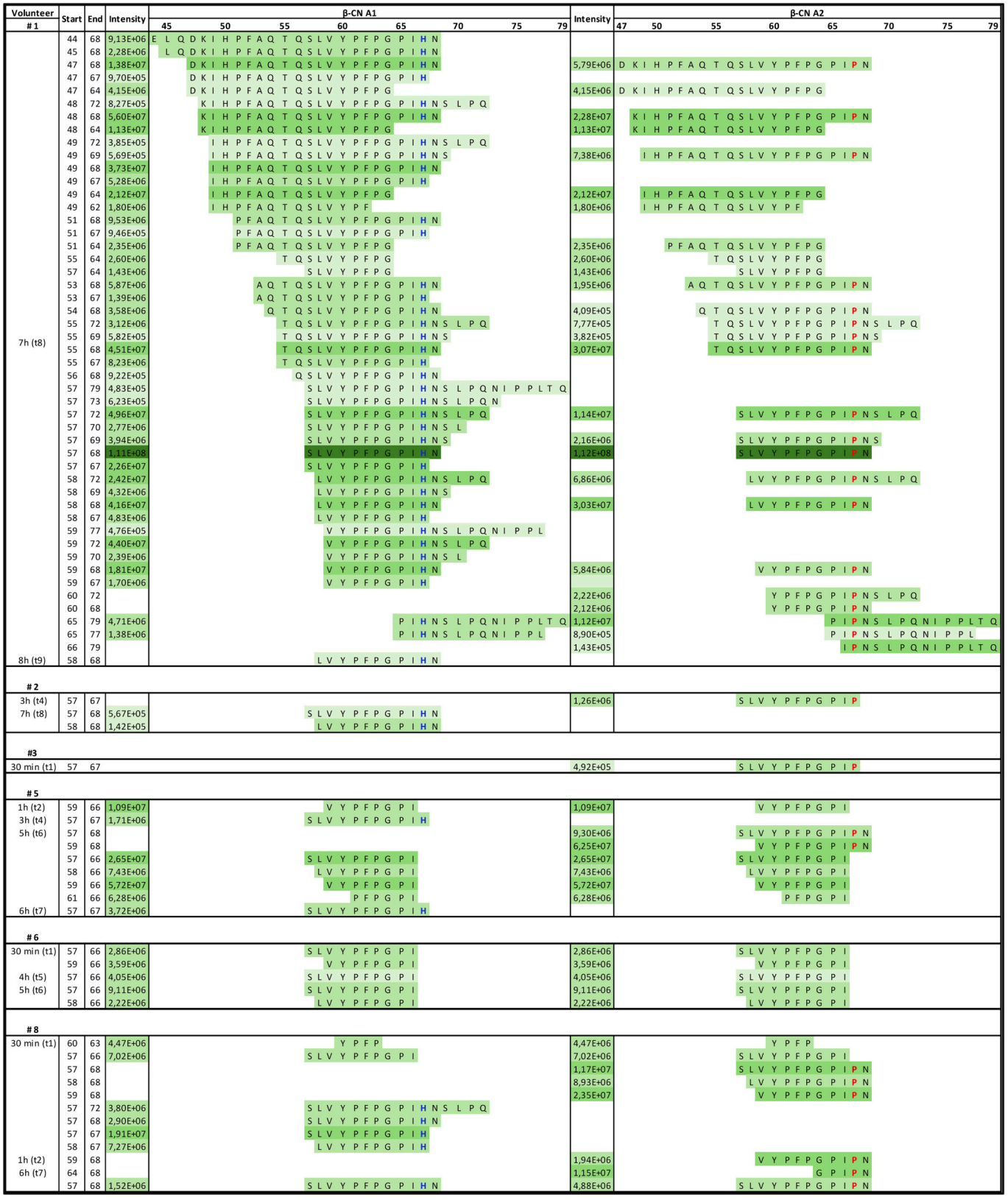

Fig. 2. Peptides associated with the BCM region of β-CN A1 and β-CN A2 were identified in the blood of volunteers #1 to #8 at various time points after the administration of 250 mL of UHT bovine milk. The data reported in the figure are derived from Supplementary Tables S1-S8. The proline and histidine residues at position 67 are highlighted in color. The timing of the peptidomic observation in the blood and the volunteer number are also reported. Peptide intensity is shown on the left side of each panel and highlighted on the identified molecule according to a green color gradation, with the dark and light shades corresponding to more and less intensity, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

32 in #3, 57 in #5, 34 in #6, 7 in #7, 108 in #8, and 0 in #4 (**Supplementary Tables S1-S8** and Table 1). Several peptides containing the BCM sequence, carrying His^{67} (from β-CNA1) and Pro^{67} (from β-CNA2), were detected in volunteer #1 (38 and 19 peptides including His^{67} and Pro^{67}, respectively), #2 (2 and 1 peptides including His^{67} and Pro^{67}, respectively), #3 (0 and 1 peptide including His^{67} and Pro^{67}, respectively), #5 (1 and 2 peptides including His^{67} and Pro^{67}, respectively), and #8 (4 and 4 peptides including His^{67} and Pro^{67}, respectively); no peptides including the BCM sequence were detected in the remaining three volunteers (# 4, #6 and #7). Additional BCM-associated peptides elongated at the N-terminal end and truncated at Ile^{66} were also identified in volunteers #5, #6, and #8. Taken together, these results demonstrated that BCM7, BCM6, and BCM5 were absent in all the blood samples examined, whereas various BCM precursors of different sizes coexisted (**Supplementary Tables S1-S8** and Fig. 2).

Fig. 2 provides quali-quantitative insights into the occurrence of BCM precursors in the blood of all volunteers, revealing a slightly greater numerical representation of fragments derived from β-CNA1. Despite potential differences in the ionization properties of His^{67}- and Pro^{67}-containing molecules, the overall intensity of the $[M + H]^+$ ions of the BCM precursors reflected their abundance and resilience against blood peptidases. The calculated ratios of the cumulative $[M + H]^+$ signal intensities for the β-CNA1- and β-CNA2-associated peptides (Fig. 2) were 1.96, 0.56, 0.63, and 0.62 for volunteers #1, #2, #5, and #8, respectively. Compared with the levels of intact β-CN variants in the milk used for the intervention (*i.e.*, β-CNA1/β-CNA2 ratio equal to 0.56) (**Supplementary Fig. S1**), these values suggested a comparable stability of BCM precursors from both β-CN variants against the action of blood peptidases in volunteers #2, #5, and #8; conversely, they indicated a slightly increased permanency of β-CNA1-derived BCM precursors only in volunteer #1, possibly due to an augmented degradation of β-CNA2-derived counterparts.

3.4. Investigating aminopeptidase-mediated degradation of BCM precursors in the blood

Food-derived peptides can be hydrolyzed before and during small intestine absorption by both *endo-* and exopeptidases, and post-absorption by plasma peptidases (Miner-Williams, Stevens, & Moughan, 2014). The sequence ladders of milk-derived peptides found in volunteers' blood allowed us to infer information on the action of exopeptidases on BCM precursors, potentially leading to BCM7 formation. Volunteer #1 presented the greatest multiplicity of BCM precursors carrying ^{65}Pro-Ile-Pro-Asn^{68} or -^{65}Pro-Ile-His-Asn^{68} C-terminal motifs (Fig. 2). These BCM precursors underwent progressive N-terminal truncation by plasma aminopeptidases, releasing various amino acids; this reiterated process could have potentially proceeded up to Tyr^{60}, yielding opioid peptides. However, peptidomic analysis revealed that BCM precursors did not persist beyond one hour in human blood, and no BCM7, BCM6, or BCM5 were detected. The sole exception was β-CN (f58–68)His^{67}, which survived for two hours before hydrolysis. These findings suggest that β-CNA1- and β-CNA2-derived BCM precursors underwent enzymatic hydrolysis without generating BCM7. Although less informative, the analysis of blood samples from volunteers #2, #3, #5, #6, and #8 (Fig. 2) confirmed that BCM precursors spanning sequences 57–68 underwent complete hydrolysis within a few hours without producing BCM7 and did not persist in plasma at 8 and 24 h post-ingestion. These observations revealed the progressive enzymatic degradation of BCM precursors in human blood.

Aminopeptidase N (APN) (3.4.11.2) is likely the primary enzyme responsible for systematically shortening the BCM precursors at the N-terminal end. However, the observed release of Leu-Gln, Ile-His, and Pro-Phe dipeptides from the N-terminus of BCM precursors suggests the concomitant involvement of dipeptidyl peptidases. DPP IV (EC 3.4.14.5), a member of the prolyl oligopeptidase family, has been identified as the primary degrading enzyme of BCM7, cleaving

Table 2
Enumeration of peptides sharing identical C-terminal amino acids identified in the blood samples of eight volunteers administered 250 mL of UHT bovine milk. The dataset encompasses BCM precursors derived from β-CNA1 and β-CNA2.

	β-CNA1-derived BCM precursors						
Numbering	66	67	68	69	70	71	72
C-term	I	H	N	S	L	P	Q
Peptides	11	12	18	4	2		7
Total peptides	54						

	β-CNA2-derived BCM precursors						
Numbering	66	67	68	69	70	71	72
C-term	I	P	N	S	L	P	Q
Peptides	11	2	18	2			4
Total peptides	37						

dipeptides from the molecular N-terminus near Pro (Fiedorowicz et al., 2014; Jarmołowska et al., 2019; Kreil, Umbach, Brantl, & Teschemacher, 1983). The concomitant action of APN and DPP IV in cleaving the BCM-spanning region at the peptide N-terminal end has recently been demonstrated through *ex vivo* experiments on synthetic BCM7 with human blood (De Pascale et al., 2024). This concerted peptidase activity was supported in this study by the concomitant detection of β-CN (f45–68)His^{67}, β-CN (f47–68)His^{67}, β-CN (f49–68)His^{67}, β-CN (f51–68) His^{67}, and β-CN (f53–68)His^{67} in volunteer #1; of β-CN (f57–66), β-CN (f58–66) and β-CN (f59–66) in volunteers #5 and #6; of β-CN (f57–67) His^{67}, β-CN (f58–67)His^{67} and β-CN (f59–67)His^{67} in volunteers #1 and #8; of β-CN (f57–68), β-CN (f58–68) and β-CN (f59–68) from both protein variants in volunteers #1 and #8; and of β-CN (f60–68)Pro^{67}, and β-CN (f60–72)Pro^{67} in volunteer #1 (Fig. 2). Although not directly detected, it cannot be excluded that BCM7 might have formed and rapidly degraded in the blood of volunteers, considering its estimated half-life of 30–35 min in human blood (De Pascale et al., 2024). Despite the untargeted character of the peptidomic approach used here and its possible limitations in detecting short peptides, the discovery of β-CN (f60–63) (BCM4) in volunteer #8 might represent the sole, indirect evidence of the transient formation and cleavage of BCM7 in blood (Fig. 2).

3.5. Deciphering C-terminal processing of BCM precursors

The peptide patterns shown in Fig. 2 also highlighted the significant involvement of carboxypeptidases in shortening BCM precursors at their C-terminal amino acids, which might be crucial for generating BCM7. The sequence ladders of BCM precursors in the blood of volunteer #1 pinpointed decisive information. Less informative but consistent results were observed for volunteers #2, #3, #5, #6, and #8, (Fig. 2). A progressive shortening of BCM precursors in the region 67–79 was detected in all individuals, resulting in peptides with C-terminal Ile^{66} in volunteers #5, #6 and #8. A comparison of BCM precursors with C-terminal amino acids 67 or 68 revealed differences between β-CNA1- and β-CNA2-derived peptides; β-CNA1-derived peptides had a comparable number of molecular species with the C-terminal signatures ^{65}Pro-Ile-His^{67} and ^{65}Pro-Ile-His-Asn^{68}, whereas β-CNA2-derived peptides predominantly presented the ^{65}Pro-Ile-Pro-Asn^{68} motif (Fig. 2, Table 2). The numerical disparity between BCM precursors containing C-terminal ^{66}Ile-His^{67} (12 peptides) or ^{66}Ile-Pro^{67} (2 peptides) motifs suggested a slightly greater stability of the C-terminal His^{67} to plasma carboxypeptidases or preferential removal of the C-terminal Pro^{67}. This phenomenon may be associated with the pK_a of the imidazole side chain (pKa = 6.0), which renders histidine a slightly worse substrate for carboxypeptidases at the pH value of blood. However, the presence of BCM precursors with both C-terminal His^{67} or Pro^{67}, together with the identification of molecules bearing C-terminal Ile^{66}, indicated the potential for both β-CNA1- and β-CNA2-derived peptides to generate BCM7.

Differences between volunteers were derived from the corresponding measured cumulative MS signal intensities of β-CNA1- and β-CNA2-derived BCM precursors with C-terminal amino acids 67 or 68. The

Table 3
Synthetic BCM precursors used in *ex vivo* proteolytic experiments with human blood. Peptides are reported according to their acronym and the corresponding molecular mass value.

Name	Synthetic Peptide	Sequence	Measured molecular mass (MH^+)
P1	(f60-68)His^{67}	YPFPGPIHN	1041.51
P2	(f60-68)Pro^{67}	YPFPGPIPN	1001.51
P3	(f60-67)His^{67}	YPFPGPIH	927.47
P4	(f60-67)Pro^{67}	YPFPGPIP	887.46
P5	(f57-68)His^{67}	SLVYPFPGPIHN	1340.70
P6	(f57-68)Pro^{67}	SLVYPFPGPIPN	1300.70
P7	(f58-68)His^{67}	LVYPFPGPIHN	1253.67
P8	(f58-68)Pro^{67}	LVYPFPGPIPN	1213.67
P9	(f59-68)His^{67}	VYPFPGPIHN	1140.58
P10	(f59-68)Pro^{67}	VYPFPGPIPN	1100.57

higher intensity values observed for β-CNA1-associated peptides in volunteer #1 (68% β-CNA1 and 32% β-CNA2, **Supplementary Table S1**) might depend on preferential degradation of β-CNA2-derived BCM precursors. In contrast, cumulative intensity values of β-CNA1- and β-CNA2-derived BCM precursors compatible with the levels of intact protein variants in milk were recorded for volunteers #2 (36% β-CNA1 and 64% β-CNA2, **Supplementary Table S2**), #3 (45% β-CNA1 and 55% β-CNA2, **Supplementary Table S3**) and #8 (35% β-CNA1 and 65% β-CNA2, **Supplementary Table S8**). Overall, these findings emphasize the differential carboxypeptidase-mediated breakdown patterns of BCM precursors in different volunteers and the relevance of specific C-terminal residues as substrates of plasma peptidases.

3.6. *Ex vivo incubation of synthetic BCM7 precursors with human blood*

Ex vivo incubation of synthetic BCM precursors of different sizes and carrying signature His^{67} or Pro^{67} (Table 3) with blood samples drawn from fasting volunteers provided insights into the degradation of these molecules by plasma peptidases. Our observations during relatively short incubation periods (up to 2 h) revealed rapidly changing peptide patterns (**Supplementary Tables S9-S18**). Regardless of the presence of His^{67} or Pro^{67}, complete peptide proteolysis within 30 min was observed for the short synthetic molecular pairs β-CN (f60–67) and β-CN (f60–68), whereas residual amounts of unhydrolyzed compounds were detected after 2 h for the larger synthetic counterparts β-CN (f59–68), β-CN (f58–68) and β-CN (f57–68). The degradation patterns of BCM precursors were similar between the two blood samples used for *ex vivo* incubation, likewise to what was previously reported for BCM7 (De Pascale et al., 2024). PCA of the ion abundance of proteolytic products from all synthetic BCM precursors within the blood of the two volunteers (Fig. 3) explained approximately 84.7% of the variance, showing parallel peptide degradation kinetics and temporal variability with characteristic peptide profiles at 1 min, 10–30 and 45–120 min of incubation.

Fig. 4 shows the average degradation pattern of each synthetic peptide and the formation of the corresponding proteolytic products. The relative intensity of the fragment peptides formed from each BCM precursor, represented by the intensity of the green shaded bars in Fig. 4, suggested a dominant action of aminopeptidases at the molecular N-terminus. Carboxypeptidases might act relatively slowly, allowing the formation of BCM7 only after His^{67} or Pro^{67} is removed. BCM7 formed from precursors β-CN (f60–67) and β-CN (f60–68) as well as from β-CN (f59–68) and β-CN (f58–68), regardless of His^{67} or Pro^{67}, but not from β-CN (f57–68) His^{67} and β-CN (f57–68) Pro^{67}. This finding indicated that longer BCM precursors at the N-terminus do not predominantly generate peptides with N-terminal Tyr^{60}. In contrast, the endopeptidase action of prolyl oligopeptidase (POP) (De Pascale et al., 2024) becomes effective in generating C-terminal Pro-containing oligopeptides, such as β-CN (f57–65), (f57–63), (f57–61), (f60–65), (f60–63), (f60–61), (f62–65), and (f64–65), irrespective of His^{67} or Pro^{67}, as shown by their relative intensities. Similar proteolytic events also seemed to occur *in vivo*, as demonstrated by the formation of the complementary peptides β-CN (f60–63) and β-CN (f64–68) in volunteer #8 seven hours after milk intake (Fig. 2). The formation of BCM7 appeared slower from synthetic His^{67}-containing BCM precursors than from their Pro^{67}-containing counterparts (**Supplementary Tables S9-S18**), which aligns with the slightly increased stability of the β-CNA1-derived BCM precursors observed in volunteer #1 (Fig. 2).

Overall, these findings emphasize the potential role of peptide length and the activity of plasma DPP IV and POP in processing the BCM precursors.

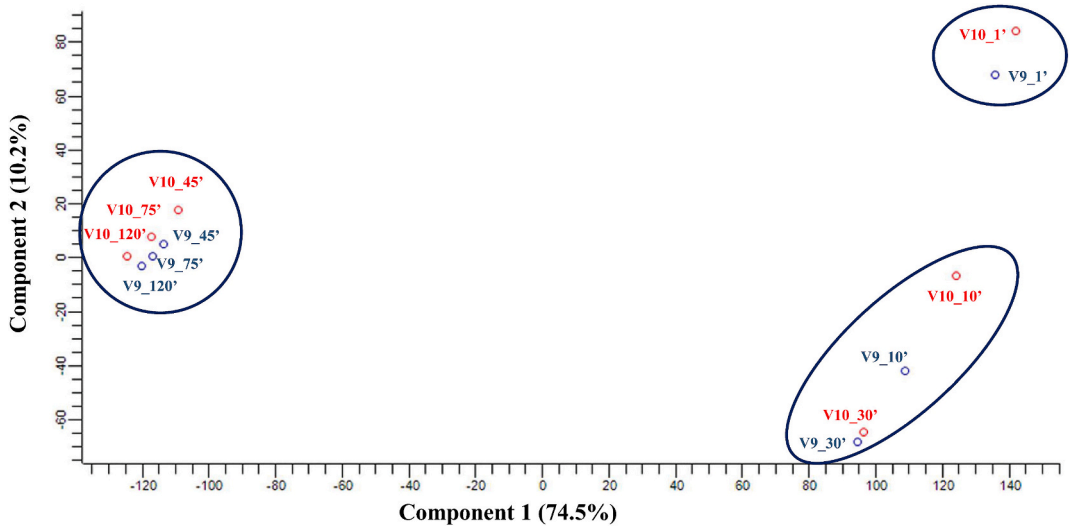

Fig. 3. Principal component analysis (PCA) for the time-course release of degradation peptide products from all synthetic BCM precursors after *ex vivo* incubation with the blood of two human individuals, referred to as volunteers #9 and #10. The names of the different points in the figure indicate the volunteer (V9 and V10) and the sampling times (T1, T10, T30, T45, T75, and T120).

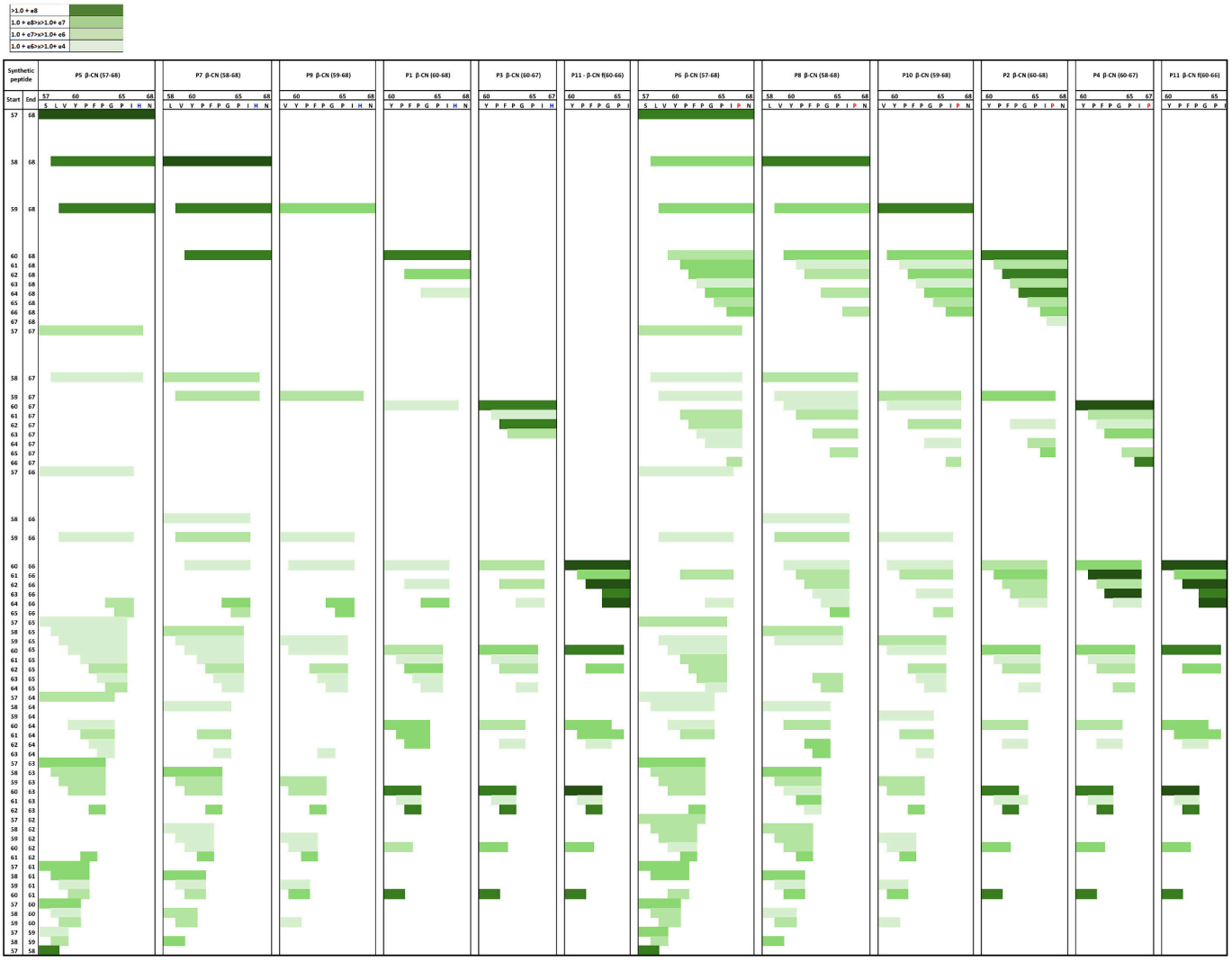

Fig. 4. Peptidomic profiles illustrating the conversion of synthetic His^{67}- and Pro^{67}-containing BCM precursors into active BCMs and non-opioid fragments after *ex vivo* incubation with the blood of volunteers #9 and #10. Information on His^{67}- and Pro^{67}-containing BCM precursors is reported on the left and right, respectively, and is referred to as information on BCM7, as derived from De Pascale et al. (2024). Data referring to each BCM precursor are reported in a dedicated column and are averaged with respect to the values of individual volunteers described in Supplementary Tables S9-S18. The size of the horizontal bar with respect to the sequence of the parent synthetic peptide denotes the nature of the β-CNA1- and β-CNA2-associated peptide fragments. The intensity of each peptide fragment is shown on the corresponding bar according to a green color gradation, with the dark and light representations corresponding to more and less intense species, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.7. Insights into the mechanisms governing the release of BCMs

Ex vivo incubation of the precursors with human blood (**Supplementary Tables S9-S18**) revealed degradation processes and the release of peptide fragments. The maximum size of synthetic BCM precursors able to generate BCM7 was eleven residues, specifically, the portion encompassing region 58–68, regardless of His^{67} or Pro^{67} (Fig. 5A). In fact, synthetic β-CN (f57–68) His^{67} and β-CN (f57–68) Pro^{67} did not yield BCM7. Differences in the production of BCM7 were recorded between homologous precursors bearing His^{67} or Pro^{67}, with Pro^{67}-containing peptides generally exhibiting slightly higher intensities and earlier opioid compound generation.

Interestingly, all the synthetic BCM precursors generated the opioid BCM5 (Fig. 5B), including β-CN (f57–68)His^{67} and β-CN (f57–68)Pro^{67}, albeit at variable intensities and with distinct kinetics. Synthetic precursors spanning the region 60–68 yielded high levels of BCM5 even after 1 min of incubation with blood, which declined and disappeared at 30 and 45 min. In contrast, the precursors spanning the region 57–68 showed consistent opioid peptide production after 10 min of incubation, which persisted for up to 2 h and probably survived even beyond this time.

The formation of BCM7 appeared to depend on both the length of the BCM precursors and the presence of His^{67} or Pro^{67}. Synthetic β-CN (f58–68)His^{67}, β-CN (f58–68)Pro^{67}, β-CN (f59–68)His^{67}, β-CN (f59–68) Pro^{67}, β-CN (f60–68)His^{67}, β-CN (f60–68)Pro^{67}, β-CN (f60–67)His^{67}, and β-CN (f60–67)Pro^{67} were all able to yield BCM7, but not β-CN (f57–68) His^{67}, and β-CN (f57–68)Pro^{67}. These findings challenge the notion that the release of BCM7 is determined only by the presence of His^{67} (Jinsmaa & Yoshikawa, 1999; Kamiński et al., 2007), highlighting the significant role of BCM precursor size in opioid peptide generation. Paradoxically, BCM precursors containing Pro^{67} generally exhibited slightly greater BCM7 production than those bearing His^{67}. In the case of BCM5, all the synthetic peptides yielded the opioid peptide.

Taken together, these findings suggest that the supposed harmful effects of bovine milk caused by BCM7 might arise from β-CN itself rather than from specific protein variants. However, these insights pertain to mechanisms governing the release of BCMs from their molecular precursors in the bloodstream and not those related to β-CN

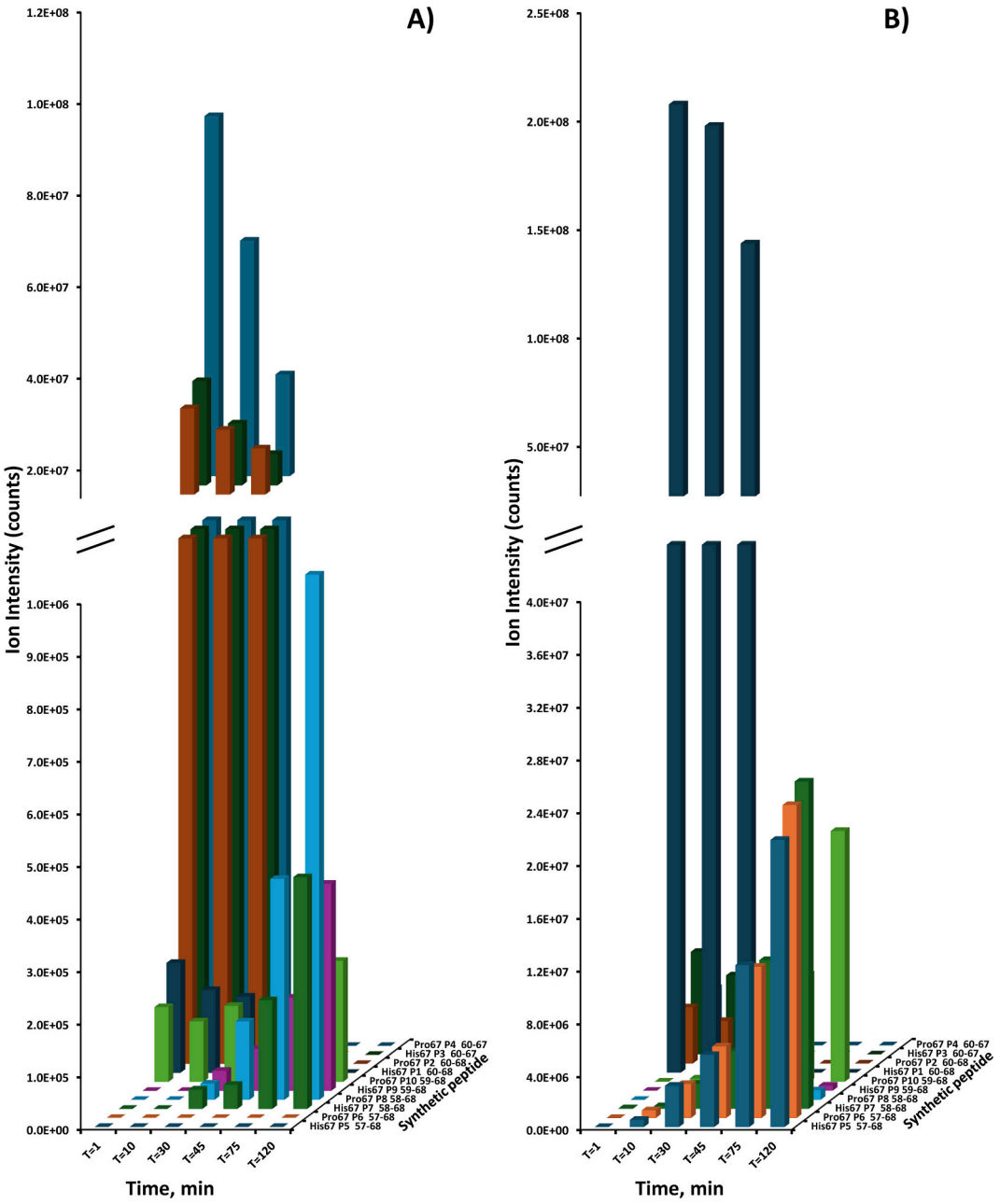

Fig. 5. Histogram illustrating the generation of BCM7 (panel A) and BCM5 (panel B) after *ex vivo* incubation of synthetic His^{67}- and Pro^{67}-containing BCM precursors with the blood of volunteers #9 and #10. Data referring to each BCM precursor are reported in a dedicated row of both graphs. The height in the histograms represents the average intensity value resulting from the data of both volunteers, as described in **Supplementary Tables S9-S18**. The sampling times (T1, T10, T30, T45, T75, and T120) are also reported.

proteolysis in the gastrointestinal tract.

4. Conclusions

BCMs were not detected in the blood of volunteers who consumed UHT bovine milk containing both β-CNA1 and β-CNA2, while significant amounts of variably sized BCM precursors from both protein variants were detected. The patterns of milk peptides varied between individuals, with His^{67}-containing BCM precursors showing slightly greater resistance to proteolysis. *Ex vivo* incubation of synthetic peptides mimicking His^{67}- or Pro^{67}-containing BCM precursors with human blood revealed that BCM5 formed independently of substrate size, whereas BCM7 was released only from the smaller substrates. Therefore, the length of the circulating BCM precursors, rather than the presence of His^{67} or Pro^{67}, seems to influence the release of BCM7. In the future, these findings could be generalized to larger cohorts of individuals, also belonging to varying population groups, strengthening their relevance.

The appearance of BCM7 in the blood does not exclusively presuppose its formation in the gastrointestinal tract. Instead, BCM precursors entering the bloodstream can be converted into BCMs depending on their length. The impact of β-CNA1 and β-CNA2 variants on the presence of BCM7 in the blood appears minimal. Given its transient nature and short half-life (30–35 min) in circulation, BCM7 may lack physiological significance, particularly in healthy individuals. At present, it is not known whether the levels of BCMs might increase in individuals with impaired barrier functionality or enzyme deficiency in the blood, potentially inducing pathological effects. The outcomes of this study indicate that the safety profile of conventional milk should not differ

from that of recently developed A2 milk. Nevertheless, based on the knowledge of release mechanisms in humans, strategies can be developed to mitigate the formation of BCM7, promoting safer practices for dairy consumption.

CRediT authorship contribution statement

Simonetta Caira: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Antonio Dario Troise:** Validation, Supervision, Formal analysis. **Gianluca Picariello:** Writing – review & editing, Writing – original draft, Validation, Supervision. **Sabrina De Pascale:** Methodology, Formal analysis. **Gabriella Pinto:** Writing – review & editing, Writing – original draft, Visualization, Validation. **Marcella Pesce:** Visualization, Validation, Supervision. **Francesca Marino:** Visualization, Validation, Supervision. **Giovanni Sarnelli:** Writing – original draft, Visualization, Validation, Supervision. **Andrea Scaloni:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Funding acquisition. **Francesco Addeo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.140477.

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