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SCIENTIFIC COOPERATION AND ASSISTANCE

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Review of the potential health impact of β-casomorphins and related peptides [1]

Report of the DATEX Working Group on β-casomorphins

(Question N° EFSA-Q-2008-379)

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WORKING GROUP MEMBERS

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SUMMARY

Proteins are a very diverse family of large organic compounds involved in many important biological processes. Following their enzymatic hydrolysis during food processing or digestion, proteins may release fragments from their primary amino acid sequence. These fragments are called peptides, and many of them are known to be physiologically active. The possible beneficial effects of bioactive peptides have attracted increasing interest in recent years. On the other hand, there are also reports suggesting that some food-derived peptides might adversely affect human health. Among these, β-casomorphin-7 (BCM7), a peptide sequence present in the milk protein β-casein, has been suggested to contribute to an increased risk for certain non-communicable diseases, such as autism, cardiovascular diseases and type I diabetes. Some literature reports have proposed possible mechanistic explanations for such associations

Recognising the alleged negative effect of BCM7 on human health, EFSA deemed it necessary to perform a comprehensive review of the published scientific literature in order to assess the relationship of this peptide and related peptides with non-communicable diseases.

The review covers the following aspects: possible sources of β-casomorphins (BCMs) and related peptides, polymorphism of β-casein, presence of BCMs and related peptides in food before digestion, formation of BCM7 and related peptides during human digestion and the possible molecular interactions of these peptides with the host environment. Furthermore, it covers the absorption and fate of these peptides, including their possible transfer mechanisms across the intestinal epithelium, transport in the blood stream and transfer across the blood-brain barrier. Finally, possible and suggested organ- and system-specific effects are reviewed, with specific emphasis on the gastrointestinal, central nervous and cardiovascular systems and the suggested link with type 1 diabetes mellitus.

This review recognises that proteins, including those present in the diet, are a potential source of a wide range of biologically active peptides, including some with affinity to opioid receptors. The latter are also known as opioid peptides. Opioid peptide sequences have been characterised in animal and plant proteins. To date much work has focused on characterising opioid peptides derived from milk proteins, in particular the caseins. Beta-casomorphins are a group of opioid peptides which can be released from β-casein. The β-casein derived peptide with the sequence Tyr^{60}-Pro^{61}-Phe^{62}-Pro^{63}-Gly^{64}-Pro^{65}-Ile^{66} is known as β-casomorphin-7.

The release of BCM7 through enzymatic digestion of bovine β-casein is dictated by different amino acid sequences of this protein. The sequences vary genetically between cow breeds. The amino acid present in position 67 of the sequence in β-casein appears to be critical for the release of BCM7. In the A^2 variant of β-casein a Proline residue occurs at position 67, whereas the A^1 and B variants of β-casein have a Histidine residue at this position. In the case of the variants containing Proline the enzymatic hydrolysis of the Ile^{66}-Pro^{67} bond does not occur or occurs at a very low rate.

The proportion of the different protein variants expressed in the milk, including those of β-casein, is related to their allele distributions in the various cattle breeds and populations. Changing selection targets in the last decades has resulted in changes in bovine breed

composition in most European countries. While no detailed information is available, it is likely that these changes in breed composition have had an impact on milk composition, including protein variants. Taking into account the lack of detailed knowledge in milk protein variant composition and the diverse geographical origin of dairy products and ingredients across Europe, insufficient information is currently available on the exposure of individual consumers to different β-casein variants.

It would appear that fresh raw/unprocessed milk obtained from healthy cows does not contain BCM7 or related peptides. By contrast, there is a substantial body of evidence indicating that different proteolytic systems involved in fermented milk or cheese manufacture can potentially hydrolyze β-casein to BCM7 or other BCMs and further degrade these peptides to shorter-chain peptides and even amino acids. However, little or no information is currently available about the actual BCM levels which different proteolytic systems may generate in fermented or enzyme-treated milk products. Furthermore, the stability of these peptides in the food products, once generated, is variable. Technological conditions, such as heat treatments, applied in industrial dairy processing do not seem to influence the occurrence of BCMs in final products or influence their formation during subsequent digestion. Moreover, limited information is available on the occurrence of BCMs in commercial infant formulas.

The role of proteolytic systems in the release of BCMs during simulated gastrointestinal digestion (SGID) or *in vivo* digestion, has not been fully clarified. No current studies report quantitative values for the formation of BCMs during *in vivo* digestion of dairy products. However, there are indications that the sequential action of several digestive enzymes may be involved and the formation of certain BCMs after SGID (with multiple enzyme activities) has been demonstrated.

Animal data clearly indicate that BCMs, including BCM7, can act as opioid receptor agonists, probably acting via μ-type opioid receptors. However, in most if not all animal studies to date, *in vivo* opioid effects for BCM7 and related milk-derived peptides have only been observed following intra-peritoneal (i.p.) or intra-cerebro-ventricular (i.c.v.) administration. In comparison to medicinal and endogenous opioids, bovine BCM7 does not seem to be a very potent opioid ligand.

A prerequisite for opioid activity after oral ingestion is that the peptides must pass the intestinal epithelial barrier. In addition, subsequent biotransformation in the liver and stability in plasma may be factors determining the ultimate biological activity. Finally, passage through the blood-brain-barrier is in principle needed for an activity in the central nervous system. Relatively little is known on the mechanisms of transfer of intact peptides longer than 3 amino acids across the intestinal barrier. If this transport occurs, then the extent is very low and passive diffusion is the most likely transfer mechanism. The presence of β-casomorphin immunoreactive material has been reported in blood in two studies with neonatal dogs and calves. However, the presence of intact β-casomorphin molecules in blood after intake of milk or casein has not been established in *in vivo* studies. Opioid peptides, including β-casomorphin 4, -5 and -7 are highly sensitive to hydrolysis by dipeptidyl peptidase IV thereby strongly limiting or preventing the transfer of these peptides in an intact form across the intestinal mucosa and the blood-brain barrier. Available data suggest that in principle,

transport of food-derived peptides and proteins across the human intestinal mucosa is possible. However, quantitative data on this phenomenon are lacking. In certain cases such as in neonates and adults with specific diseases, intestinal permeability has been reported to be significantly increased. In general, the review did not find any quantitative data on the absorption of intact bioactive peptides for adults, except in the case for di- and tripeptides with reported antihypertensive properties.

Food-derived peptides, including casomorphins, can have different effects in the intestinal lumen and the intestinal mucosa, such as regulatory effects on gastro-intestinal motility and on gastric and pancreatic secretions. Many studies report effects of β-casomorphins on the central nervous system (CNS) following i.p. or i.c.v. administration in animals. A possible link between BCM intake and sudden infant death syndrome (SIDS) has been suggested in some publications. However, no clear evidence for such a relationship was found during the review. The mechanisms proposed were considered rather speculative and partly contradictory. Similarly, a link between casein-derived peptides and autism in subjects with increased intestinal permeability has been suggested. However, recent data do not provide any support for such a relationship.

It has been suggested that BCM7 might be atherogenic through an oxidative action on LDL. This mechanism was originally proposed in a single report; however, it has not been confirmed by later studies. By contrast, numerous *in vitro* studies indicate that many food derived peptides/hydrolysates display antioxidant activity. The possibility that BCM7 could contribute to an increased risk for atherosclerosis has also been suggested from a study in a rabbit model. This study concluded that β-casein A^1 would be atherogenic, in contrast to β-casein A^2. However, during the review the validity of the experimental model and the extrapolation to atherosclerosis in humans were not regarded as convincing. Further speculations for an association between BCM7 intake and cardiovascular disease mortality have been raised as a result of ecological studies. However, these ecological studies did not account for several confounding factors. In addition, recent large cohort studies led to opposite conclusions. Two human intervention studies comparing diets containing β-casein A^1 and A^2 did not show a correlation between the estimated β-casein A^1 consumption and development of certain biomarkers for cardiovascular disease (CVD). A limitation of these studies was the small number of subjects and the short intervention period. Overall, this review process did not find any strong evidence for a link between the consumption of β-casein A^1 and an increased risk for CVD in humans.

Insulin dependent diabetes mellitus (IDDM) is recognised as a multifactorial autoimmune disease; however, its pathogenesis is unclear. Autoantibodies have been found in IDDM patients, but the role of autoantibodies in type 1 diabetes is currently not known. These autoantibodies have not been proven to be directly involved in either tissue destruction or disease progression. The development of IDDM is the result of a combination of genetic predisposition and environmental risk factors where genetic predisposition is a necessary condition.

Many dietary risk factors of IDDM have been indicated, including short duration of breast feeding, as well as administration of gluten, soy, cow's milk and solid foods at young age.

Several different milk proteins or peptides derived from these proteins have been proposed as possible diabetogenic factors. The mechanism most often suggested is immunological. The diabetogenicity of β-casein A^1, A^2 and B has been evaluated in animal studies and ecological studies on humans. Animal studies have presented contradictory results.

Some ecological studies have linked the intake of BCM7 with IDDM. Ecological studies have the shortcoming of being unable to establish a cause-effect association and they cannot adjust for possible confounding factors. They are at best indicating a hypothesis but cannot provide a proper base to demonstrate a cause-effect relationship.

The correlations suggested by such studies may become very weak when taking into account the uncertainties in individual consumption, in the β-casein variant composition, and in some countries also the IDDM incidence rate.

Moreover, the difference in content of β-casein A^1+B in milk produced in countries with high or low prevalence of IDDM appears relatively small and does not explain, from an immunological point of view, the difference in incidence of IDDM across countries.

Based on the present review of available scientific literature, a cause-effect relationship between the oral intake of BCM7 or related peptides and aetiology or course of any suggested non-communicable diseases cannot be established. Consequently, a formal EFSA risk assessment of food-derived peptides is not recommended.

Key Words: food-derived peptides, opioid peptides, β-casein, β-casomorphin, β-casomorphin-7, IDDM, CVD, SIDS, autism

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BACKGROUND

Proteins are a broad family of large organic compounds playing a central role in the structure and functionality of all living organisms. Besides their structural and functional roles proteins are also a fundamental component of animal and human diets providing a source of energy, nitrogen and essential amino acids. In addition, dietary proteins may also provide a source of biologically active peptides. Such peptides are inactive within the sequence of the precursor protein but may be released from their respective animal, plant or microbial proteins by hydrolysis during food processing or digestion. Food-derived bioactive peptides may influence regulatory functions in the human system beyond normal nutrition. A range of food protein derived peptides have been found to be potentially physiologically active, i.e., bioactive. These bioactivities include modulation of gut secretion and motility, blood pressure-lowering, antithrombotic, antioxidant, antimicrobial and immunomodulatory activities. Some of these effects are mediated by interaction with the opioid system (Mine and Shahidi, 2006).

On the one hand the discovery of bioactive peptides with potential health benefits has been the subject of growing commercial interest in the context of health-promoting functional foods. To date milk proteins have been most extensively studied as a source of these peptides. The opioid receptor system is of particular interest since it has been proposed as one of several possible links between physiological effects and the intake of food protein derived peptides. Food derived peptides which bind to opioid receptors are known as exogenous opioid peptides. Some evidence exists, for example, to indicate that these peptides may beneficially modulate several gastrointestinal functions (Teschemacher, 2003). Milk proteins, including both caseins and whey proteins are a source of many peptides with opioid activities, including casomorphins and lactorphins. The *in vivo* physiological effects of many of these peptides, however, remain to be fully elucidated.

On the other hand, there are reports of potential adverse health effects of food-derived opioid peptides. Some ecological studies have suggested that the milk derived peptide BCM7 (BCM7) or related compounds may be involved in the development of a diverse range of serious human diseases, such as juvenile diabetes type I, ischemic heart disease, autism and schizophrenia (Elliott *et al.*, 1999; McLachlan, 2001). A number of different biological mechanisms have been suggested for the adverse effects of BCM7 and related compounds, based on their opioid, lipid oxidation (Torreilles *et al.*, 1995), histamine release (Kurek *et al.*, 1992) and immunological activities (Padberg, 1999; Pozzilli, 1999). The possible release of BCM7 during protein digestion is generally reported to be dependent on the presence of specific genetic variants of β-casein, specifically variants A^1 and B of bovine β-casein (Hartwig, 1997; Jimsmaa *et al.*, 1999).

TERMS OF REFERENCE

To collect and review the available scientific evidence related to BCM7 and analogous peptides with similar characteristics, with the purpose to assess the existence and robustness of an association between such bioactive peptides originating from food and non-communicable diseases. The detailed objectives include:

- Possible occurrence of BCM7 or related peptides as a function of genetic variability of the food source;
- Factors leading to their formation, absorption in the intestinal tract and metabolic pathways;
- Identification of possible specific vulnerability of particular sub-populations;
- Mechanisms of action of these peptides in the body, including immunological effects;
- A critical assessment of epidemiological evidence of adverse health effects in humans;
- A critical evaluation of experimental evidence in animals fed with diets leading to high levels of BCM7 or analogous peptides.

Following this review and depending on its outcome EFSA will decide whether there are sufficient indications to justify a full risk assessment of the consumption of food that can potentially produce such bioactive peptides during digestion. Should the review identify a lack of information on which to base a reasonable decision the need for any further research will be highlighted.

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ASSESSMENT

Different approaches are possible to assess the robustness of an alleged association between consumption of a substance and non-communicable diseases. Nevertheless, some common criteria may be identified to test a cause-effect hypothesis (WHO, 2008; US-FJC, 2000). These include

- *temporal relationship;*
- *strength of the association;*
- *dose–response relationship;*
- *substantial convergence of the studies (replication of the findings);*
- *biological plausibility (including mechanisms of transport and mechanisms of action);*
- *consideration of alternative explanations;*
- *effects on cessation of exposure;*
- *specificity of response*
- *consistency with other knowledge.*

The present review takes into account these suggestions, focusing on different relevant aspects:

- Possible sources of β-casomorphins and related peptides
- Polymorphism of β-casein
- Presence of β-casomorphins and related peptides in food before digestion
- Formation of β-casomorphins and related peptides during human digestion
- Possible molecular interactions of these peptides
- Absorption and fate of these peptides, including transfer mechanisms across the intestinal epithelium, transport in the blood stream and transfer across the blood-brain barrier
- Organ and system-specific suggested action of food-derived peptides, focusing on the gastrointestinal system, the central nervous system, the cardiovascular system and type 1 diabetes mellitus

1 INTRODUCTION

Proteins are a very broad family of large organic compounds playing a central functional and structural role in living organisms. Chemically proteins are linear polymers built from different L-α-amino acids. All 20 amino acids possess common structural features, including a α carbon to which an amino group, a carboxyl group, and a variable side chain are bonded. Only proline differs from this basic structure as it contains an unusual ring to the N-end amine group, which forces the CO–NH amide moiety into a fixed conformation. The side chains of the standard amino acids have different chemical properties that produce a three-dimensional protein structure and different activities. The amino acid sequence is therefore of critical importance for the protein functionality/biofunctionality.

The amino acids in a polypeptide chain are linked by peptide bonds. Once linked in the protein chain, an individual amino acid is called a residue, and the linked series of carbon, nitrogen, and oxygen atoms are known as the main chain or protein backbone. The peptide bond has two resonance forms that contribute some double-bond character and inhibit rotation around its axis, so that the α-carbons are roughly coplanar. The other two dihedral angles in the peptide bond determine the local shape assumed by the protein backbone.

Due to the chemical structure of the individual amino acids, the protein chain has directionality. The end of the protein with a free carboxyl group is known as the C-terminus or carboxy terminus, whereas the end with a free amino group is known as the N-terminus or amino terminus. Amino acids in a polypeptide chain are numbered starting from the N-terminus.

The sequence of amino acids in a protein is crucial to the role it plays in a living organism, and each protein is defined by a gene and encoded in the genetic code.

Different terms are used to define linear sequences of amino acids, such as protein, polypeptide, and peptide. The meaning of these terms can sometimes overlap, however, protein is generally used to refer to the complete biological molecule in a stable conformation, whereas peptide is generally reserved for short amino acid oligomers. The boundary between the two terms is not well defined and is usually considered at about 20–30 residues. Polypeptide is a general term for any single linear chain of amino acids, usually regardless of length.

Besides their roles in the structure and functionality of the organism, proteins are also a fundamental component of animal and human diet providing a source of energy, nitrogen and essential amino acids. To this purpose dietary proteins need to undergo a breakdown process known as digestion, which is carried out by many different hydrolytic enzymes that progressively lead to the formation of peptides and free amino acids. Enzymes are site-specific in their cleavage action on the polypeptide chain, some being more selective than others. Depending on the enzymes involved, digestion of a single dietary protein may involve formation of different patterns of intermediate peptides.

2 BIOACTIVE PEPTIDES AND SOURCE PROTEINS

2.1 Protein precursors for bioactive peptides

Globally, public health professionals in particular, but also consumers, food producers and food processors, are becoming increasingly aware of the rapidly expanding body of epidemiological evidence linking the prevalence of diseases, such as cardiovascular disease, obesity, hypertension, diabetes and even cancer to dietary factors. This has led to an increased interest in the potential health effects of food derived bioactive components. Food proteins contain various peptide sequences encrypted within their primary structures which possess potential biological activity. These proteins originate from a range of different sources including animals, fish, plants and bacteria (Mine and Shahidi, 2006). These bioactive peptides may be released from their parent protein molecule either during food processing and/or during gastrointestinal digestion.

Depending on their site of action, these peptide sequences may or may not be required to be transported across the intestinal mucosa in order to mediate a physiological response. Such bioactive peptides range in size from 2 to 50 amino acid residues and may have numerous physiological functions within the body. Peptides, for example, having hypotensive, immunomodulatory, antibacterial, mineral binding, antithrombotic along with opioid agonist and antagonist activities have been reported. The literature on milk protein derived bioactive peptides has been extensively reviewed (Clare and Swaisgood, 2000; FitzGerald and Meisel, 2003; Korhonen and Pihlanto, 2003; Pihlanto and Korhonen, 2003; Meisel, 2004; Walsh and FitzGerald, 2004; Korhonen and Pihlanto, 2006; Gobbetti *et al.*, 2007; Hartmann and Meisel, 2007; Korhonen and Pihlanto, 2007; Murray and FitzGerald, 2007; Morris and FitzGerald, 2008). The literature indicates that these peptides potentially impact the cardiovascular, nervous, digestive and immune systems (Figure 1).

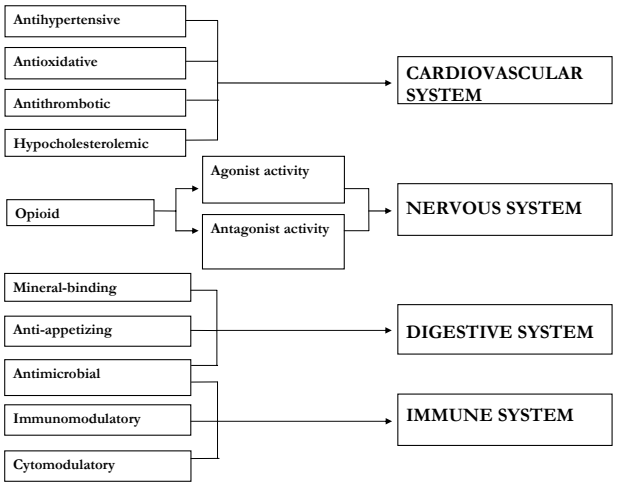

Figure 1: Potential physiological functionality of food-derived bioactive peptides (adapted from Korhonen and Pihlanto, 2007)

In order to become active in the body it is considered very critical that a bioactive peptide first reaches its molecular target. This subject will be further discussed in chapter 4.4.

2.2 Food protein sources of opioid peptides

Many different food protein molecules contain peptide sequences which behave as opioid receptor ligands (Henschen *et al.*, 1979, Zioudrou *et al.*, 1979, Brantl *et al.*, 1986a, Paroli 1988, Teschemacher 2003, Kostyra *et al.*, 2004, Guesdon *et al.*, 2006). It has been reported that opioid peptides can be formed from milk, cereal, vegetable and meat/poultry proteins (Table 1). Furthermore, various blood proteins, including albumin and γ-globulins (Zioudrou *et al.*, 1979) and haemoglobin (Brantl *et al.*, 1986b) may act as a source of opioid peptides. In addition, the egg protein, ovalbumin also appears to contain opioid peptide sequences (Zioudrou *et al.*, 1979).

Table 1: Food protein sources of opioid receptor ligands/peptides (adapted from Teschemacher, 2003)

Food Group	*Foodstuff*	*Protein precursor*	*Reference*
Dairy	Milk and milk products	α-Casein	(Zioudrou *et al*., 1979)
		β-Casein	(Brantl *et al*., 1979)
		κ-Casein	(Chiba *et al*., 1989)
		α-Lactalbumin	(Yoshikawa *et al*., 1986)
		β –Lactoglobulin	(Yoshikawa *et al*., 1986)
		Lactoferrin	(Tani *et al*., 1990)
Cereals	Wheat	Gluten	(Fukudome and Yoshikawa, 1992)
		Gliadin	(Zioudrou *et al*., 1979)
	Barley	Hordein	(Zioudrou *et al*., 1979)
		Avenin	(Zioudrou *et al*., 1979)
		Secalin	(Zioudrou *et al*., 1979)
		Zein	(Zioudrou *et al*., 1979)
	Rice	Albumin	(Takahashi *et al*., 1994)
Vegetable	Soy	α-Protein	(Zioudrou *et al*., 1979)
	Spinach	Rubisco	(Yang *et al.,* 2003)
Meat/poultry	Blood	Albumin	(Zioudrou *et al*., 1979)
		Haemoglobin	(Brantl *et al.,* 1986b)
		γ-Globulin	(Zioudrou *et al*., 1979)
	Egg	Ovalbumin	(Zioudrou *et al*., 1979)

Food protein derived opioid peptides are classified as exogenous opioids in that while they possess a Tyr residue within their sequence, usually at the N-terminus or in the N-terminal region, they differ from the endogenous opioid peptides which often have Tyr-Gly-Gly-Phe as the N-terminal sequence (Teschemacher, 2003). In most cases these exogenous peptides have been isolated and subsequently identified from enzymatic digests of their parent protein molecules. However, in some cases, synthetic peptides corresponding to specific regions within intact food protein molecules have been shown to act as opioid receptor ligands.

Milk proteins are the most studied with respect to the presence of opioid peptide sequences and have been extensively reviewed in the literature (Meisel, 1997; Teschemacher *et al.,* 1997; Meisel and FitzGerald, 2000; Pihlanto-Leppälä, 2000; Guesdon *et al.,* 2006). Most studies have been performed with bovine milk proteins, but there are a limited number of studies on opioid peptides from water buffalo (Petrilli *et al.*, 1984) and from human milk (Brantl, 1984; Koch *et al.*, 1985; Ferranti *et al.*, 2004).

As can be seen from Table 1, all the major milk proteins contain opioid receptor ligands. These have been specifically termed exorphins and casoxin D when derived from α-casein, β-casomorphins and β-casorphins when derived from β-casein, casoxins (A, B, C) when derived from κ-casein, α-lactorphins when derived from α-lactalbumin, β-lactorphins when derived from β-lactoglobulin and lactoferroxins when derived from lactoferrin. The majority of the

milk peptides identified to date behave as opioid agonists while the casoxins and lactoferroxins behave as opioid antagonists.

Table 2 provides specific details of opioid peptide sequences from different food protein sources.

Table 2: Parent protein precursors and associated opioid peptide sequences from different food protein sources.

Origin	Structure
Bovine Milk	
α-lactorphin, α -la f(50-53)	Tyr-Gly-Leu-Phe (YGLF)
β-lactorphin, β-lg f(102-105)	Tyr-Leu-Leu-Phe (YLLF)
α_{S1}-casein-exorphin, αs1-cn f(90-96)	Arg-Tyr-Leu-Gly-Tyr-Leu-Glu (RYLGYLE)
β-casomorphin 4, β-cn f(60-63)	Tyr-Pro-Phe-Pro (YPFP)
β-casomorphin 5, β-cn f(60-64)	Tyr-Pro-Phe-Pro-Gly (YPFPG)
β-casomorphin 7, β-cn f(60-66)	Tyr-Pro-Phe-Pro-Gly-Pro-Ile (YPFPGPI)
β-casomorphin 8, β-cn f(60-67)	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro/His (YPFPGPIP/H)
Casoxin A, κ-cn f(35-42)	Tyr-Pro-Ser-Tyr-Gly-Leu-Asn-Tyr (YPSYGLNY)
Casoxin B, κ-cn f(58-61)	Tyr-Pro-Tyr-Tyr (YPYY)
Casoxin C, κ-cn f(25-34)	Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu-Ser-Arg (YIPIQYVLSR)
serorphin, BSA f(399-404)	Tyr-Gly-Phe-Asn-Ala (YGFNA)
Bovine Blood	
hemorphin-4, β-chain of bovine haemoglobin, f(34-37)	Tyr-Pro-Trp-Thr (YPWT)
hemorphin-5, β-chain of bovine haemoglobin, f(34-38)	Tyr-Pro-Trp-Thr-Gln (YPWTQ)
hemorphin-7, β-chain of bovine haemoglobin, f(34-40)	Tyr-Pro-Trp-Thr-Gln-Arg-Phe (YPWTQRF)
LVV-hemorphin-7, β-chain of bovine haemoglobin, f(31-40)	Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe (LVVYPWTQRF)
V-hemorphin-5 hemorphin-7, β-chain of bovine haemoglobin, f(34-40)	Val-Tyr-Pro-Trp-Thr-Gln (VYPWTQ)
VV-hemorphin-7, β-chain of bovine haemoglobin, f(32-40)	Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe (VVYPWTQRF)
Human milk	
Casoxin D, α_{S1}-cn f(158-164)	Tyr-Val-Pro-Phe-Pro-Pro-Phe (YVPFPPF)
β-casomorphin (1–4), β-cn f(51-54)	Tyr-Pro-Phe-Val (YPFV)
β-casomorphin (1–5), β-cn f(51-55)	Tyr-Pro-Phe-Val-Glu (YPFVE)
β-casomorphin (1–7), β-cn f(51-57)	Tyr-Pro-Phe-Val-Glu-Pro-Ile (YPFVEPI)
β-casomorphin (1–8), β-cn f(51-58)	Tyr-Pro-Phe-Val-Glu-Pro-Ile-Pro (YPFVEPIP)
β-casorphin (1–4), β-cn f(41-44)	Tyr-Pro-Ser-Phe (YPSF)
Goat milk	
α_{S1}-cn f(90-95) variant B	Arg-Tyr-Leu-Gly- Tyr-Leu (RYLGYL)
α_{S1}-cn f(90-96) variant B	Arg-Tyr-Leu-Gly- Tyr-Leu-Glu (RYLGYLE)
α_{S1}-cn f(91-96) variant B	Arg-Tyr-Leu-Gly- Tyr-Leu-Glu (YLGYLE)
κ-cn f(25-34)	Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu-Ser-Arg (YIPIQYVLSR)
κ-cn f(33-38)	Ser-Arg-Tyr-Pro-Ser-Tyr (SRYPSY)
κ-cn f(35-38)	Tyr-Pro-Ser-Tyr (YPSY)
κ-cn f(35-41)	Tyr-Pro-Ser-Tyr-Gly-Leu-Asn (YPSYGLN)
κ-cn f(56-61)	Leu-Pro-Tyr-Pro-Tyr-Tyr (LPYPYY)
κ-cn f(58-61)	Tyr-Pro-Tyr-Tyr (YPYY)
κ-cn f(56-61) variant J	Leu-Pro-Tyr-Pro-Tyr-Cys (LPYPYC)
κ-cn f(58-61) variant J	Tyr-Pro-Tyr-Cys (YPYC)
Sheep milk	

Origin	Structure
β-casomorphin 8, β-cn f(60-67)	Tyr-Pro-Phe-Thr-Gly-Pro-Ile-Pro (YPFTGPIP)
Buffalo milk	
β-casomorphin 7, β-cn f(60-66)	Tyr-Pro-Phe-Pro-Gly-Pro-Ile (YPFPGPI)
Cereal	
Gluten exorphin A4	Gly-Tyr-Tyr-Pro (GYYP)
Gluten exorphin B4	Tyr-Gly-Gly-Trp (YGGW)
Gluten exorphin A5	Gly-Tyr-Tyr-Pro-Thr (GYYPT)
Gluten exorphin B5	Tyr-Gly-Gly-Trp-Leu (YGGWL)
Gliadorphin 7 (also known as gluteomorphin)	Tyr-Pro-Gln-Pro-Gln-Pro-Phe (YPQPQPF)
Gluten exorphin C5	Tyr-Pro-Ile-Ser-Leu (YPISL)
Vegetable	
Rubiscolin-5	Tyr-Pro-Leu-Asp-Leu (YPLDL)
Rubiscolin-6	Tyr-Pro-Leu-Asp -Leu-Phe (YPLDLF)
Soymorphin 5	Tyr-Pro-Phe-Val-Val (YPFVV)

Adapted from Meisel and FitzGerald, 2000; Yang *et al.*, 2001; Teschemacher, 2003; Chessa *et al.* 2008.

2.2.1 Bioinformatic screening

Using a bioinformatics approach it is possible to identify other potential biological precursors for opioid peptides. For this review the NCBI RefSeq (http://www.ncbi.nlm.nih.gov/ accessed October 2008) and the Swiss-Prot (http://www.ebi.ac.uk/swissprot/ accessed October 2008) databases were searched for protein precursors which contain the following exogenous opioid peptides sequences:

- YPFPG: β-casomorphin 5
- YPFP: β-casomorphin 4
- YPFVV: Soymorphin 5
- YGGWL: Gluten exorphin B5
- YPLDLF: Rubiscolin **6**

The results of this bioinformatic search are presented in Table 3. This table does not include the well known food proteins summarized in Table 2.

Table 3: Summary of bioinformatic search for less common parent protein precursors containing selected opioid peptide sequences.

Organism	Function or Name	Sequence	Reference
YPFPG (β-casomorphin 5)			
Synechococcus	Enzyme	(173)-YPFPG-(177)	Swissprot, Q2JK10, Q2JY06
Mus musculus Homo sapiens (Human)		(452)-YPFPG-(455)	Swissprot Q8K243, Q6AZZ1
E. coli	Oxido-reductase	(398)-YPFPG-(402)	Swissprot

			P37906
Canis familiaris (Dog). Homo sapiens (Human).	Glycogen-debranching enzyme	(50)-YPFPG-(54)	Swissprot P35573, Q2PQH8
Oryctolagus cuniculus (Rabbit).	Glycosyltransferase	(73)-YPFPG-(77)	Swissprot P35574
Homo sapiens (Human).	Protein Kinase	(100)-YPFPG-(104)	Swissprot P49842
YPFP (β-casomorphin 4)			
Saccharomyces cerevisiae	Putative uncharacterized protein oligosaccharyl transferase	(76)-YPFP-(79) (338)-YPFP-(341)	Swissprot P87283 P48439
Homo sapiens (Human).	Transmembrane protein	(143)-YPFP-(146)	Swissprot Q5BJD5
Gallus gallus (Chicken)	Transmembrane protein	(121)-YPFP-(124)	Swissprot Q5ZIL6
Homo sapiens (Human) Mus musculus	Tenascin	(1978)-YPFP-(1981) (1887)- YPFP-(1890)	Swissprot P24821, Q80YX1
Drosophila melanogaster	Protein split ends	(1708)-YPFP-(1711)	Swissprot Q8SX83
Salmo salar (Atlantic salmon)	Probable signal peptidase complex subunit 2	(80)-YPFP-(83)	NCBI ACI69108
Homo sapiens (Human).	rna exonuclease	(944)-YPFP-(947)	Swissprot Q8N1G1
Bos taurus (Bovine).	Lipid transport	(105)-YPFP-(108)	Swissprot P02720
YPFVV (Soymorphin)			
Homo sapiens (Human). Mus musculus	Mismatch repair endonuclease	(318)-YPFVV-(322)	Swissprot P54278, P54279
Gibberella zeae	FACT complex subunit POB3	(305)-YPFVV-(309)	Swissprot Q4IJU0
Arabidopsis thaliana	Serine/threonine-protein kinase	(1872)-YPFVV-(1876)	Swissprot Q9FKS4
Gallus gallus (Chicken)	Aprataxin	(59)-YPFVV-(63)	Swissprot P61798
YGGWL (Gluten exorphin B5)			
Homo sapiens (Human). Mus musculus Rattus norvegicus	Myosin phosphatase Rho-interacting protein	(46)-YGGWL-(50)	Swissprot Q6WCQ1, P97434, Q9ERE6
Arabidopsis thaliana	Sugar transport protein	(461)-YGGWL-(465)	Swissprot Q8L7R8
YPLDLF (Rubiscolin 6)			

Vitis vinifera	Ribulose bisphosphate carboxylase large chain	(103)-YPLDLF-(108)	Swissprot P56648
Synechococcus sp. Spinacia Oleracea	Ribulose bisphosphate carboxylase large chain	(95)-YPLDLF-(100) (103)-YPLDLF-(108)	Swissprot Q0I8Q2, P00875
Solanum tuberosum	Ribulose bisphosphate carboxylase large chain	(103)-YPLDLF-(108)	Swissprot P25079

As can be seen from Table 3, sequences corresponding to opioid peptides were found in diverse proteomes from animals, humans, microbes and plants. The significance of the presence of opioid peptides in these proteomes is unclear. However, given the right hydrolytic conditions the human proteome itself could act as a source of peptides which interact with the opioid system. For opioid sequences detected in proteomes other than the human proteome, it is necessary to take into account the presence of these proteins in food derived from these organisms (e.g. salmon, chicken, *Saccharomyces cerevisiae*) and the proteolytic system involved in their breakdown.

2.3 Significance of genetic polymorphism to the release of opioid peptides from β-casein

The primary sequence of all food proteins is subject to genetic variation. This is true also in the case of bovine milk proteins. Furthermore, genetic polymorphism may dictate the type of bioactive peptides released from milk proteins. For example, in β-casein an amino acid substitution occurs at position 67 in different variants

…-Tyr^{60}-Pro^{61}-Phe^{62}-Pro^{63}-Gly^{64}-Pro^{65}-Ile^{66}-(His^{67})-… β-casein A^1 and B variants

…-Tyr^{60}-Pro^{61}-Phe^{62}-Pro^{63}-Gly^{64}-Pro^{65}-Ile^{66}-(Pro^{67})-… β-casein A^2 variant

In β-casein A^1 and B variants histidine occurs in position 67, whereas in β-casein A^2 proline is present in the same position.

This genetic substitution of histidine with proline has been reported to prevent the enzymatic hydrolysis of the peptide bond between residues 66 and 67 in β-casein A^2 thereby preventing the release of BCM7 (β-casein f(60-66)) from β-casein A^2. (Hartwig, 1997; Jinsmaa *et al.*, 1999)

Since genetic polymorphism is breed related, much interest has focused on characterising β-casein variability in bovine populations, particularly when looking at possible health effects of BCMs.

The first evidence of genetic polymorphism in β-casein came from Aschaffenburg (1961) while studying milk from Jersey and Guernsey cows. Using paper electrophoresis in the presence of 6.0 M urea in citrate-phosphate buffer pH 7.5, he reported that β-casein existed as three polymorphs, designated in order of decreasing mobility as β-casein A, B and C.

Subsequently, Peterson and Kopfler (1966), using acid urea polyacrylamide gel electrophoresis discovered that β-casein A could be separated into three additional variants, now known as β-casein A^1, A^2 and A^3. These variants differ from each other by the number of positively charged His residues in the primary sequence, i.e., 6, 5 and 4, respectively. More recently, the application of chromatographic separation, isoelectric focusing and DNA-based techniques has led to the discovery of a range of other β-casein polymorphs. The specific amino acid substitutions which distinguish some of the different β-casein variants are summarised in Table 4.

Table 4: Amino acid substitutions in some genetic polymorphs of bovine β-casein (adapted from Ng-Kwai-Hang and Grosclaude, 2003).

Variant	Amino acid position, amino acid substitution
$A^1 \rightarrow A^2$	^{67} His → Pro
$A^2 \rightarrow A^3$	^{106} His → Gln
$A^1 \rightarrow B$	^{122} Ser → Arg
$A^1 \rightarrow C$	^{37} Glu → Lys ^{35} SerP → Ser
$A^2 \rightarrow D$	^{18} SerP → Lys
$A^2 \rightarrow E$	^{36} Glu → Lys

Numerous studies have been performed on the allele frequency distributions of the three main species of the genus *Bos*, i.e. *Bos taurus* (taurines), *Bos indicus* (zebu) and *Bos grunniens* (yak). However, to date most studies have been performed on taurines as they are the main milk producing cattle breed in the Western world. A non-exhaustive summary of the reported breed and country related frequency distributions for taurine β-caseins is outlined in Table 5.

Table 5: Summary of β-casein allele frequency distribution in selected Western cattle breeds.

Breed	No. Animals	β-Casein Allele Frequency				Reference
		A^1	A^2	B	Other	
Angus	77 (USA)	0.95		0.05		Caldwell *et al.*, 1971
Ayrshire	45 (USA)	0.72	0.28			Kiddy *et al.*, 1966
Ayrshire	29 (UK)	0.60	0.40			Aschaffenburg, 1968
Ayrshire	? (Canada)	0.60	0.40			Ng-Kwai-Hang and Kim, 1994
Ayrshire	37 (N. Zealand)	0.432	0.527			Winkelman and Wickham, 1997
Ayrshire	20,990(Finland)	0.509	0.490	0.001		Ikonen *et al.*, 1996
Ayrshire	46 (Finland)	0.50	0.50			Lien *et al.*, 1999
Black Pied	140 (Estonia)	0.97*		0.03		Pupkova, 1980
Braham	59 (USA)	0.99*		0.01		Caldwell *et al.*, 1971
Brown Italian	298 (Italy)	0.11	0.69	0.18	0.02	Boettcher *et al.*, 2004
Brown Swiss	? (Canada)	0.32	0.52	0.16		Ng-Kwai-Hang and Kim, 1994
Brown Swiss	22 (USA)	0.14	0.66	0.18	C: 0.02	Kiddy *et al.*, 1966
Brown Swiss	50 (USA)	0.18	0.66	0.16		Van Eenennaam and Medrano, 1991
Canadienne	? (Canada)	0.58	0.34	0.08		Ng-Kwai-Hang and Kim, 1994
Dutch-Fr	10151 (Holland)	0.766	0.147	0.014	B:0.073	Bovenhuis and van Arendonk, 1991
Finncattle (EFC)	31 (Finland)	0.274	0.710	0.016		Lien *et al.* 1999
Finncattle (NFC)	26 (Finland)	0.385	0.615			Lien *et al.* 1999
Finncattle (WFC)	41 (Finland)	0.293	0.671	0.037		Lien *et al.* 1999
Friesian(Fr)	3761 (N. Zealand)	0.465	0.510			Winkelman and Wickham, 1997
Friesian(Fr)	347 (Italy)	0.38	0.55	0.07		Boettcher *et al.*, 2004
Grey	120 (Hungary)	0.23	0.76	0.01		Baranyi *et al.*, 1993
Guernsey	196 (USA)	0.01	0.98	0.02		Aschaffenburg, 1963
Guernsey	40 (USA)		0.96		C:0.04	Van Eenennaam and Medrano, 1991
Hereford	48 (USA)	0.75*		0.25		Caldwell *et al.*, 1971
Holstein	260 (Australia)	0.63	0.35	0.02		McLean *et al.*, 1984
Holstein	1383 (Italy)	0.58	0.40	0.02		Aleandri *et al.*, 1997
Holstein	1152 (USA)	0.43	0.55	0.02		Van Eenennaam and Medrano, 1991
Holstein-Fr	85 (UK)	0.66	0.24	0.06	A^3:0.04	Aschaffenburg *et al.*, 1968
Holstein-Fr	87 (Germany)		0.960	0.040		Aschaffenburg *et al.*, 1968
Holstein-Fr	6460 (Canada)	0.54	0.44	0.01	A^3:0.01	Hines *et al.*, 1977
Holstein-Fr	6575 (USA)	0.42	0.53	0.02	A^3:0.03	Hines *et al.*, 1977
Holstein-Fr	260 (USA)	0.624	0.347	0.025	A^3:0.004	McLean *et al.*, 1984

Breed	No. Animals	β-Casein Allele Frequency				Reference
		A^1	A^2	B	Other	
Holstein-Fr	920 (Canada)	0.363	0.632	0.001	A^3:0.004	Lin *et al.*, 1986
Holstein-Fr	696 (Ireland)	0.72	0.25	0.03		O' Hara, 1995
Holstein-Fr	43 (Finland)	0.430	0.523	0.047		Lien *et al.* 1999
Holstein-Fr	143 (Poland)	0.402	0.598			Kaminski *et al.*, 2007
Icelandic	44 (Iceland)	0.326	0.674			Lien *et al.*, 1999
Jersey	37 (USA)	0.22	0.49	0.29		Kiddy *et al.*, 1966
Jersey	47 (UK)	0.09	0.63	0.28		Aschaffenburg, 1968
Jersey	308 (Australia)	0.07	0.57	0.36		McLean *et al.*, 1984
Jersey	157 (Denmark)	0.07	0.58	0.35		Bech and Kristiansen, 1990
Jersey	172(USA)	0.17	0.50	0.33		Van Eenennaam and Medrano, 1991
Jersey	? (Canada)	0.19	0.50	0.31		Ng-Kwai-Hang and Kim, 1994
Jersey	116 (Ireland)	0.30	0.41	0.28	A^3 : 0.01	O' Hara, 1995
Jersey	1328 (N. Zealand)	0.123	0.591			Winkelman and Wickham, 1997
Kerry	123 (Ireland)	1.00*				Murphy and Downey, 1969
Kerry	41 (Ireland)	0.24	0.76			O' Hara, 1995
Normande	155 (France)	0.21	0.32	0.45	A^3: 0.02	Aschaffenburg, 1968
Norwegian	38(Norway)	0.513	0.487			Lien *et al.*, 1999
Red Danish	169 (Denmark)	0.71	0.23	0.06		Bech and Kristiansen, 1990
Shorthorn	40(USA)	0.49	0.49	0.02		Van Eenennaam and Medrano, 1991
Simmental	2626 (Denmark)	0.231	0.673	0.082	C : 0.013	Baranyi *et al.*, 1993
Simmental	621(Croatia)	0.190	0.630	0.150		Curik *et al.*, 1997
SLB	43(Sweden)	0.407	0.593			Lien *et al.*, 1999
SLB	42 (Sweden)	0.34	0.60	0.06		Hallén *et al.* 2008
Spotted	101 (Hungary)	0.21	0.715	0.06	C : 0.015	Baranyi *et al.*, 1993
SRB	394(Sweden)	0.460	0.531	0.008		Lunden *et al.*, 1997
SRB	39(Sweden)	0.397	0.603			Lien *et al.*, 1999
SRBH	39 (Sweden)	0.44	0.55	0.01		Hallén *et al.* 2008
SRBL	35 (Sweden)	0.36	0.63	0.01		Hallén *et al.* 2008
White Danish	223 (Denmark)	0.55	0.39	0.03	A^3 : 0.03	Bech and Kristiansen, 1990

* the β-casein A genetic variants were not distinguished into A^1, A^2 and A^3 variants

? number of animals in study not given; EFC – Eastern Finncattle; NFC – Northern Finncattle; WFC – Western Finncattle

As can be seen from Table 5, the most commonly occurring β-casein variants in Western cattle breeds are A^1, A^2 and B. Country related differences in the β-casein allele frequencies are observed which may be reflective of local breeding policies, some cross-breeding and most importantly targeted breeding for increased milk production traits. Some interesting trends arise from the data. For example, Jersey, Normande and Simmental breeds appear to consistently have a relatively low frequency of occurrence of β-casein A^1. In comparison with the other breeds listed in this table Jersey, Normande and Hereford have a relatively high frequency of occurrence of the B variant of β-casein. On the other hand, Guernsey cattle almost exclusively appear to have the β-casein A^2 genotype. The Brown Swiss, Brown Italian, Hungarian Spotted and Gray breeds had a relatively higher occurrence of β-casein A^2 compared to A^1 alleles. The relative distribution of β-casein A^1 and A^2 alleles in Holstein and Friesian breeds appears to be region/country specific with certain regions having a high level of β-casein A^1, others having high level of A^2 and others again having roughly similar allele frequency distributions for A^1 and A^2 (Table 5).

However, it is important to take sample size into account, as in Table 5 where population sizes tested range from approximately 20 to 21,000 animals.

2.4 Variation in β-casein variant frequency distribution as a function of time

Data in Table 5 cover a 40-year period and give us a general view of the allele frequency distribution in different cattle breeds only at the respective time of analysis. It is thus difficult to draw definitive conclusions on the relative distribution of β-casein variant proteins in milk for a given consumer population at any given time.

Breeding of bovine cattle has been ongoing in Europe for centuries with the objective to increase both carcass weight and milk yield. As an example, in the Nordic countries milk yield has increased significantly between 1985 and 1997 (van Arendonk and Liinamo, 2003) and correspondingly a significant change in the breed composition of national herds has taken place. Comparative data are available for Finland, where before the 1960's milk production was largely based on Finncattle (Northern, Eastern and Western) while nowadays it is mainly from Finnish Ayrshire and Finnish Holstein-Friesian (Liinamo, 2000). Similarly, the breed composition of the milk-producing cattle in other European countries has changed during the same time period.

Therefore, given the β-casein allele frequency distribution differences between breeds (Table 5) it may be assumed that in countries where herd composition has changed over the years there have been corresponding changes in the β-casein variant composition in the milk.

However, in certain countries such as Iceland, where the importation of foreign cattle breeds has not been allowed, it may be assumed that the breed composition of the milk producing cattle has not changed significantly in the same way. Genetic variation in such cases is also possible, though expectably slower, due to the selection practices employed within the breed to maintain or improve its traits. However, to our knowledge no direct data is available about changes in the β-casein variant composition of milk in Icelandic cattle over time.

In addition, apart from some studies in selected Nordic countries in the late 1990's (Lien *et al.*, 1999; Elliott *et al.*, 1997, Thorsdottir *et al.*, 2000, Iggman, 2003) we do not have any specific Europe wide information on the β-casein variant composition of bulk milk currently produced in Europe.

With globalisation in the marketplace we have little direct information or indeed control over the origin of the milk proteins in market milk, in fermented dairy products or in formulated foods incorporating milk protein ingredients. Therefore, the protein variant composition of milk and milk ingredients, and the overall level of intake of specific milk protein variants by different populations is currently unknown.

2.5 Polymorphism in human milk

Human milk varies in protein content ranging from 1.4 – 1.6 g/100 ml during early lactation and declining to about 0.7 – 0.8 g/100 ml at 6 months of lactation (Lönnerdal *et al.*, 1976; Kunz *et al.*, 1992). The proportion of casein to total protein in human milk ranges from 10% at early lactation to approximately 40% in mature milk (Kunz and Lönnerdal, 1990; 1992). The overall composition of human milk is dependent on the stage of lactation along with specific variations in the mother's genotype. β-casein is the major casein component in human milk (Greenberg *et al.*, 1984) however, κ- (Brignon *et al.*, 1985) and α_{S1}-casein are also present in low amounts (Cavaletto *et al.*, 1994; Rasmussen *et al.*, 1995). Very little appears to be known about genetic variation in human milk caseins. However, the results of Dev *et al.* (1994) as interpreted by Steinerova *et al.* (2004) seem to indicate that human milk does not contain β-casein A^1.

3 OPIOID PEPTIDES IN FOOD AND THEIR FORMATION DURING DIGESTION

In the scientific literature many papers deal with the occurrence of opioid peptides in dairy foods prior to or after simulated gastrointestinal digestion (SGID). The data reported has been obtained using analytical techniques, including liquid chromatography (LC) as a peptide separation technique with UV detection and/or mass spectrometry (MS) for identification and quantification of BCMs. Different digestion protocols employing one or more gastric and intestinal proteinases have been used for the release of BCMs. It is evident that the approach used for isolation, identification and quantification of BCMs impacts on the reliability and the usefulness of results. Furthermore, additional uncertainty arises from the published data depending on whether real or model dairy food systems were used.

The theoretical yield of different opioid peptides per gram of precursor protein is summarised in Table 6.

Table 6: Precursor proteins and theoretical yield for some opioid agonist and antagonist peptides derived from cow milk proteins (adapted from Meisel and FitzGerald, 2000)

Bioactive peptide	Sequence	Precursor Protein	Maximum theoretical yield (mg/g protein)
Opioid agonists			
BCM7	YPFPGPI f(60-66)	β-cn	33.0
BCM5	YPFPG f(60-64)	β-cn	24.2
α_{s1}-Casein exorphin	RYLGYLE f(90-96)	α_{s1}-cn	38.7
α-Lactorphin	YGLF f(50-53)	α -la	35.2
β-Lactorphin	YLLF f(102-105)	β-lg	30.2
Serorphin	YGFNA f(399-404)	BSA	10.5
Opioid antagonists			
Casoxin A	YPSYGLNY f(35-42)	κ-cn	51.4
Casoxin B	YPYY f(58-61)	κ-cn	31.8
Casoxin C	YIPIQYVLSR f(25-34)	κ-cn	65.8

3.1 Peptides presence in food

3.1.1 Human milk

There are a limited number of reports on opioid peptides identified in human milk. These peptides were initially studied by Brantl *et al.* (1984) and Koch *et al.* (1985) and more recently by Ferranti *et al.* (2004). Using MALDI/TOF/MS and ESI/MS Ferranti *et al.* (2004) reported that human milk casein was more susceptible to proteolysis than bovine milk casein. Most of the low and medium molecular weight peptides in human milk collected during the first week of lactation originated from the endogenous hydrolysis of β-casein. Two opioid peptides corresponding to β-casomorphin (1–8) and β-casorphin (1–4) were identified. These authors further reported that the size of the peptides decreased as a function of increasing gestation and lactation period. In fact, while larger precursors fragments of BCMs (51–73, 48–73 and 35–73) were present both in preterm and normal term milk, the shorter sequences (including β-casomorphin 1–8) were only detected in milk from women delivering after the normal gestation period (39 and 40 weeks).

Using an HPLC/UV methodology, Jarmolowska *et al.* (2007a) determined the content of endogenous BCM5 and BCM7 in human milk during different stages of lactation. They reported that the BCM content decreased during the progression of lactation. They also reported that the BCM content of colostrum was five times higher for BCM5 and eight times higher for BCM7 compared to the content of these peptides in mature milk. The BCM5 and BCM7 levels in colostrum were in the range from 0.00 to 19.77 μg/mL and 0.00 to 26.78 μg/mL, respectively. In mature milks (2 and 4 months after delivery) the levels ranged from zero to 10.56 μg/mL and 0.00 to 2.84 μg/mL for BCM5 and BCM7, respectively.

3.1.2 Bovine milk

One study reports the presence of BCM7 in unprocessed bovine milk (Cieslinska *et al.*, 2007). Other studies confirming these findings are not available in the literature. The identification was performed using HPLC/UV methodology, and the health status of the animals from which the milk was derived was not reported. Somatic cells in milk are a normal phenomenon and their count increases dramatically during clinical and also sub-clinical mastitis. Increasing somatic cell count (SCC) is associated with increasing proteolysis of caseins. Therefore, the enhanced proteolytic activity in animals having a high SCC may contribute to the release of BCMs or their precursors. The specificity and activity of cathepsins and elastase derived from SCCs have been studied in model systems but the actual levels of secretion of these enzymes in milk itself have not been reported. The activity of cathepsin B found in lysosomes of somatic cells is significantly correlated with the SCC of milk and it has been shown that it partially survives conventional pasteurisation (O'Driscoll *et al.*, 1999). Considine *et al.* (2004), studied *in vitro* digestion of β-casein and found that the activity of cathepsin B was similar to or identical to that of plasmin and cell envelope proteinases (CEP) produced by a number of *Lactococcus lactis* strains. Furthermore, cathepsin B was reported to be able to release BCM10 (f60–69). Wedholm *et al.* (2008) studied the activities of enzymes released from somatic cells in a milk sample containing > 500.000 cells/mL corresponding to sub-clinical *Streptococcus uberis* mastitis. The results indicated that cathepsins and elastase were associated with β-casein proteolysis, however, release of BCMs was not observed.

Using MALDI MS and MS/MS, Napoli *et al.* (2007) studied the activity of the endogenous proteinases during 24- to 216-h incubation at 37 °C at both physiological and acid pH values in unprocessed milk produced from each quarter of the mammary gland of a healthy cow (SCC=0.6-1.3 x 10^3) and a mastitic cow (SCC=0.4-11.6 x 10^6). They identified β-casein derived peptides indicating cleavage sites located toward the C-terminus of the protein but no hydrolysis appeared to occur in the BCM region. Proteinases of somatic cells may also be involved in proteolysis of β-casein during storage of UHT milk, since no study to date has demonstrated thermal inactivation of these enzymes during industrial UHT processing of milk.

According to Gaucher *et al.* (2008), β-casein appeared to be hydrolyzed in UHT (140 °C for 4 sec) treated milk following storage at 20 °C for 6 months. Using LC-MS/MS, they identified different pro-BCMs (f54–68, f54–69, f55–65, f55–68, f57–68) resulting from the hydrolytic action of cathepsin B, cathepsin G and elastase on β-casein.

3.1.2.1 Fermented milk

A number of studies have reported on the formation and fate of BCM7 in fermented dairy products, including cheese, fermented milk and yoghurt. However, only a few detail the actual level of BCM7 in these dairy products. The lack of quantifiable data arises from the difficulty in identifying and quantifying BCMs in such complex food matrices which are comprised of many peptides as a result of milk protein hydrolysis. The formation/degradation of BCM7 has been mainly studied using purified caseins or synthetic BCMs as substrates and single bacterial strains or their associated proteinase/peptidase systems.

Hamel *et al.* (1985) were the first to identify β-casomorphin immunoreactive material in cow's milk following incubation with various bacterial species, including lactic acid bacteria (LAB). The proteolytic systems in LAB involve both CEP and intracellular peptidases; the former being responsible for releasing different oligopeptides from intact milk protein molecules which are further hydrolysed by the peptidases into shorter fragments and free amino acids. Recent studies with 21 *Lactobacillus* strains isolated from fermented milks have demonstrated that these LAB were able to degrade 80–90% of the β-casein following 72-96 h *in vitro* incubation of a sodium caseinate solution (Tzvetkova *et al.*, 2007). Similar findings were recorded for LAB proteinases from *Lactococcus lactis* subsp. *cremoris* Wg2 which hydrolysed approximately 40% of the peptide bonds in β-casein resulting in more than 100 oligopeptides (Mierau *et al.*, 1997; Juillard *et al.*, 1995).

In general, the formation of casomorphins in dairy products fermented with LAB has been considered unlikely given that these bacteria have a high intracellular X-prolyldipeptidyl aminopeptidase (PepX) activity (Gobbetti *et al.*, 2002). PepX is the best characterized proline-specific enzyme produced by LAB and it releases x-Pro dipeptides from the N-terminus of peptides. Hydrolysis with PepX would remove the X-Pro sequence which is central for the bioactivity of BCM7. According to Matar and Goulet (1996), the cell-free extracts of the wild type or PepX-deficient mutant strains of *Lactobacillus helveticus* L89 totally or partially hydrolyse synthetic BCM7 into BCM4 after incubation at 37°C for 120 min. Using LC/MS, the authors also reported the formation of BCM4 but not BCM7 in a pasteurized (65 °C for 30 min) milk fermented with a PepX-deficient mutant of *Lactobacillus helveticus* L89. According to Stepaniak *et al.* (1995), BCM7 inhibits the *in vitro* activity of peptidases (PepO and PepN) from *Lactococcus lactis* ssp. *lactis* MG1363. However, the PepX activity of the same microorganism was not inhibited by this peptide and PepX was actually reported to hydrolyse BCM7. Muehlenkamp *et al.* (1996) evaluated the susceptibility of synthetic BCM3, -5 and -7 to the proteolytic system of a commercial strain of *Lactococcus cremoris*. They reported after 6-15 weeks of incubation that BCM7 was more resistant to proteolysis than BCM3 and BCM5.

The type of starter used seems to affect the nature of the bioactive peptides released in fermented milks. It is well known that *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, which are used in combination as a starter in yoghurt manufacture, release peptides that promote the growth of the mixed culture. In general, due to the short fermentation time, lysis of cells of LAB is unlikely and intracellular peptidase activity is unlikely to significantly contribute to proteolysis during yoghurt manufacture (Meisel and Bockelmann, 1999). As reported by Donkor *et al.* (2007), most of the proteolytic activity in yoghurt containing *L. delbruekii* ssp. *bulgaricus* Lb1466 and *S. thermophilus* St1342 occurs during the first 24 h following manufacture and subsequently continues to increase at a slower rate during storage (28 d) at 4 °C. However, according to Schieber and Brückner (2000), storage at 4 °C for 3 weeks resulted in extensive proteolysis of milk proteins in yoghurt made from skimmed milk heated to 90 °C and fermented (44 °C for 3 h) using the above two LAB strains. In this product, the majority of the peptides arose from β-casein A^1 breakdown but only peptides, including BCM containing sequences (i.e. β-casein 57-68 and β-casein 57-72), were identified by means of HPLC-MS and peptide sequencing. Kahala *et al.* (1993) investigated the peptides

released from several Finnish fermented milk products, including two yoghurts, produced with a mixed starter culture of *L. bulgaricus* and *S. thermophilus*. Most of the peptides identified originated from either the N- or C-terminal region of β-casein, however no BCMs were found.

According to Donkor *et al.* (2007), proteolysis in fermented milk containing both yoghurt starter culture and probiotic organisms (*L. acidophilus* L10, *L. casei* L26 and *B. lactis* B94) was significantly higher as compared to the milk fermented with the yoghurt culture only. Using *in vitro* tests, Shihata and Shah (2000) reported that extracts containing proteolytic systems of yoghurt bacteria (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) were highly proteolytic compared to those from probiotic bacteria (*L. acidophilus* and *Bifidobacterium* spp.). It appears that the use of probiotic strains for production of fermented milk influences the peptide profile in the final product. Furthermore, the potential release of BCMs during gastrointestinal digestion may also be affected by the peptide profile. In this regard, a UHT (142 °C for 3-4 sec) milk fermented with the probiotic Lactobacillus GG strain which milk was subsequently digested with pepsin and trypsin was found to contain peptide sequences similar to BCM11 (derived from β-casein A^2) and BCM4 (Rokka *et al.*, 1997). These peptides were not present in the fermented milk prior to pepsin and trypsin digestion.

3.1.2.2 Cheese

The formation and fate of BCM7 have been studied in different cheese varieties which are considered a very complex food system. The extent and the pattern of proteolysis in cheese varies as a function of heat treatment of milk (unprocessed or pasteurized), type of coagulant (animal or vegetable), curd handling (cooking, salting, pH at draining), starter culture and ripening conditions (pH, time, temperature, humidity, secondary microflora). Most of the proteolysis occurs during ripening and as a result caseins are hydrolysed by endogenous proteinases, coagulant, and starter and non-starter proteinases. Moreover, as previously mentioned, BCM7 or related sequences produced by either endo- or exogenous enzymes may exert an inhibitory effect on intracellular peptidases of LAB (Stepaniak *et al.*, 1995). All the above factors influence BCM7 release/degradation which therefore must be quantified under real cheesemaking conditions. In this regard, the action of the retained chymosin itself is not expected to release BCMs in cheese, irrespective of the pH value and salt concentration of cheese (McSweeney, 2004).

Furthermore, proteolysis induced by plasmin does not lead to formation of known bioactive peptides (McSweeney, 2004). As already mentioned, both starter and non starter LAB proteinases/peptidases may hydrolyse proline-rich peptides, such as BCMs, which may be formed upon the action of proteolytic systems present in the cheese milk/curd. As peptidase activity is intracellular in LAB, it is likely that LAB peptidases contribute to protein hydrolysis only after cell lysis which generally occurs during cheese ripening (Meisel and Bockelmann, 1999). Additional proteolysis may be brought about by the action of fungal enzymes involved in the ripening of certain cheese varieties. Despite the fact that the mechanisms of action of most of the proteinases present in cheese are well elucidated, it is not possible to predict the formation/degradation of BCM7 in cheese. Only very few studies report on the presence and levels of BCM7 in cheeses. Jarmolowska *et al.* (1999), using

amino acid composition analysis reported BCM7 as being most likely present in extracts from Brie cheese samples. This peptide occurred in amounts ranging from 5 to 15 mg/kg cheese following different (not specified) maturation times. Previously, Muehlenkamp *et al.* (1996) reported no detectable amounts of BCM7 in the same cheese variety. HPLC separation coupled to UV detection was used for identification and quantitation of BCM7. In addition, BCM7 was not detected in other cheese varieties having different extents of proteolysis: Cheddar, Swiss type (60 day ripened), Blue and Limburger. Therefore, to date no data concerning the actual amounts of BCM7 in cheeses can be obtained from the literature.

Muehlenkamp *et al.* (1996) reported that BCM7 was almost resistant to degradation by the proteolytic system of *Lactococcus cremoris*. This was particularly the case at pH 5.0-5.2 and high sodium chloride concentration (5%) as employed in the *in vitro* enzymatic tests. At lower salt concentration (1.5%), BCM7 was partially (50%) or totally degraded after 15 weeks at pH 5.0 or pH 5.2, respectively. Taking the pH value (5.0-5.1) and salt concentration (1.8-2.0%) of Cheddar cheese into consideration, BCM7 is expected to be degraded to some extent by the starter culture used in Cheddar cheese manufacture. Information is available about the formation of peptides in enzyme-modified Cheddar cheese. Using LC-MS/MS, Haileselassie *et al.* (1999) identified several potentially bioactive peptides, including BCM7, from an enzyme-modified Cheddar cheese (EMC) prepared using Neutrase®, a neutral protease produced from *Bacillus subtilis*. However, the actual level of BCM7 in the EMC was not reported. The same authors did not find BCM7 when the enzyme-modified cheese was prepared using a crude extract of *L. casei* isolated from a mild Cheddar cheese. The findings of Haileselassie *et al.* (1999) suggest the destructive action exerted by proline specific peptidases from LAB on BCM7 present in cheese. As discussed later, other studies report cheeses to contain peptides including the BCM7 sequence. According to Singh *et al.* (1997), the persistence of peptides such as BCM9 (β-casein 60-68) in Cheddar suggests that PepX from LAB has no or little activity in cheese, perhaps because it is unstable.

Several studies indicate that different cheeses contain peptides incorporating the BCM7 sequence. These peptides could act as BCM7 precursors during further digestion processes. Addeo *et al.* (1992) revealed the presence of long peptides (e.g. 20-21 residues) containing the BCM7 sequence in ripened (up to 15 months) Parmigiano Reggiano cheese. According to the authors, the presence of such peptides demonstrates the resistance of this β-casein region to further enzymatic degradation. It is noteworthy that in this type of cheese extensive casein breakdown occurs during ripening at the end of which about 20% of protein nitrogen is represented by free amino acids. Stepaniak *et al.* (1995) isolated the β-casein (58-72) peptide (from genetic variant A^1) from the water-soluble extract of Cheddar and Jarlsberg cheeses. Both the 57-58 and 56-57 bonds of β-casein have been reported to be easily hydrolyzed by proteinases from LAB (Visser *et al.*, 1991). The β-casein (58-72) peptide was also released during Crescenza ripening (Smacchi and Gobbetti, 1998). This peptide was apparently resistant to further degradation by enzymes present in cheese and it was not hydrolysed by PepO and PepN activities. As already mentioned, β-casein (58-72) is reported to selectively inhibit endopeptidase (PepO), general aminopeptidase (PepN), and PepX from *Lc. Lactis* ssp. *lactis* MG1363 (Stepaniak *et al.*, 1995). BCM9 (from variant A^1 of β-casein) and several peptides including the BCM7 sequence were found in Cheddar cheese by Singh *et al.* (1995

and 1997). BCM9 was also found in Gouda (8 months aged) by Saito *et al.* (2000). Using LC-MS-TOF and LC-MS/MS, Toelstede *et al.* (2008) found BCM9 and BCM10 in the water-soluble extract of a matured Gouda cheese. BCM9 was released from the A^1 and A^2 variants of β-casein whereas BCM10 was derived from the A^1 variant. Pro-BCM9 and pro-BCM10 peptides were liberated from both variants by cleavage of Gln^{56}– Ser^{57}, Ser^{57}-Leu^{58} and Leu^{58}-Val^{59} bonds.

The proteolytic systems potentially involved during manufacture and ripening of Emmental cheese were investigated by Gagnaire *et al.* (2001). Due to inactivation of coagulant by the high curd cooking temperature, CEP from thermophilic lactobacilli, chatepsin D and plasmin were mainly thought to be responsible for the hydrolysis of β-casein from which neither BCMs nor pro-BCMs were released. The role of bifidobacteria in releasing BCM3 and BCM5 during ripening of Edam cheese was studied by Sabikhi *et al.* (2001) using HPLC/UV detection. Both Edam and probiotic Edam cheeses contained BCM3 whereas BCM5 was not present. Using LC-MS, Rizzello *et al.* (2005) analyzed the water-soluble extracts of Caprino del Piemonte, an Italian goat cheese. A peptide corresponding to *β*-CN f60–68 (YPFTGPIPN) was identified in the cheese extract.

3.1.2.3 Infant formulas

The potential occurrence of BCMs in commercial infant formulas has not been extensively studied. In the only study published to date, Jarmolowska (2007b) assayed for opioid activity in samples of “Humana” formula available on the Polish market. Four opioid peptides with agonistic or antagonistic activity were found in the peptide extract of this formula and its pepsin–trypsin hydrolysate. These peptides were identified as BCM5, casoxin C and 6 and lactoferroxin A. The opioid activity of the peptide extracts was determined by examining their influence on the motor activity of isolated rabbit intestine. The reported amounts (μg/mL) of these peptides in the peptide extract of Humana and its pepsin hydrolysate were as follows: BCM5 (0.39, 43.11), casoxin 6 (0.22, 6.21), casoxin C (0.075, 23.33) and lactoferroxin A (0.16, 11.40). Generally, the amounts of opioid peptides in the pepsin hydrolysate of "Humana" were significantly higher than in the non-pepsin treated sample. A recent paper using LC/MS reports no BCM5 or BCM7 in milk-based infant formulas (De Noni, 2008).

3.1.3 Effect of milk processing on the release opioid peptides

To our knowledge, no study has considered the effect of milk processing conditions on the release of BCMs during subsequent fermentation. It is well recognised that intense heat treatments (90-95 °C for 5-20 min) applied in yoghurt manufacturing can induce thermal modifications of protein contributing to the desired properties of the final product (i.e. viscosity, lack of syneresis). Heating causes unfolding of protein molecules and hence modifies their secondary and tertiary structure. In this regard, caseins are not as susceptible to severe heat denaturation compared to the globular whey proteins. Native β-casein appears to form only short lengths of α-helixes and has a less pronounced tertiary structure. For this reason, β-casein cannot or can hardly be denatured and its conformation is not expected to change much upon thermal treatment.

Schmelzer *et al.* (2007) studied the proteolysis of β-casein during peptic digestion under simulated gastro-intestinal digestion (SGID). No preferred pepsin cleavage sites were found on the basis of predicted secondary structure. This finding supports the hypothesis that pepsin would most likely act in a similar manner on either the native or the heated forms of β-casein. Severe heat treatment of milk strongly enhances formation of β-lactoglobulin/κ-CN stable complexes on the surface of casein micelles. These complexes are believed to reduce casein proteolysis as they make the access of proteinases more difficult (Enright *et al.*, 1999). The results obtained by Almaas *et al.* (2006) from digestion of both cows' and goats' milk with human gastric and duodenal juices showed that heated milk in general was more resistant to hydrolysis than unprocessed milk.

Covalent interactions occurring upon heating involve both lactosylation and crosslinking within or between casein chains. These reactions can result in the formation of xenobiotic molecules some of which may be responsible for aggregation/polymerisation of β-casein molecules (Pellegrino *et al.*, 1999). Deamidation and dephosphorylation of β-casein can occur when processing milk during yoghurt manufacturing (Van Boekel, 1999). The effect of these modifications of β-casein on its susceptibility to proteolysis during milk fermentation cannot be speculated. The role of thermal treatments in hindering or promoting BCM release from this protein molecule has not been elucidated, so far.

To date, no study has been published concerning the effect of UHT treatment or in-bottle sterilisation on potential release of BCMs during digestion of heat-treated milk. Milk-based infant formulas are submitted to intense heat treatment during manufacture. Moreover, they contain dairy ingredients that in turn are usually obtained by industrial processing, including severe heat treatments. Therefore, heat damage can occur in the final infant formula and a diverse degree of protein modification could be expected. In the recent study of De Noni (2008), industrial indirect UHT treatments (156 °C for 6–9 sec) were not found to modify the release of BCM7 and, during SGID comparable amounts of peptides were formed from both raw formulations and heat-treated infant formulas.

3.2 Peptide formation during *in vitro* simulated gastro-intestinal digestion (SGID)

3.2.1 Milk derived opioids released in SGID

The potential release of BCMs during gastrointestinal digestion of dairy products has been investigated in a number of studies. For this purpose, different protocols based on SGID have been adopted. The usefulness and reliability of these protocols depends on both enzymatic system and physiological factors adopted. Digestion process parameters, including the rate of gastric emptying and the changes in gastric pH value, are particularly relevant, especially in infants. In general, the use of hydrolytic enzyme preparations containing several amino and carboxyl peptidase formulations should lead to more reliable and comparable results as to what may happen during GI transit. Irrespective of the difference in protocols, it seems that an initial hydrolysis by pepsin plays a key role during SGID of β-casein as has been clearly elucidated by Jinsmaa *et al.* (1999). In this study, BCM9, -13 and -21 or (Val^{59})-BCMs were released after initial cleavage by pepsin of the Leu^{58}-Val^{59} bond in β-casein followed by

further degradation with intestinal proteinases. Very low levels of BCM7 (17 mmol BCM7/mol β-casein) were found in the elastase-leucine aminopeptidase digest of β-casein when the preliminary pepsin treatment at pH 2.0 was omitted. The role of pancreatic enzymes was also studied and no BCM7 was released when chymotrypsin or trypsin were used in place of pancreatin for β-casein digestion following pepsin treatment. Incubation with pepsin alone does not appear to release BCM7.

Macaud *et al.* (1999) using second-order derivative spectra and UV-spectra comparison detected BCM3 but not BCM7 in a pepsin hydrolysate of casein. According to Schmelzer *et al.* (2007), the release of known bioactive peptides from the A^1 or A^2 variants of β-casein following peptic digestion under SGID is unlikely. They reported no formation of BCM7 following 10-60 min digestion with pepsin at pH 2.0. β-casein degradation was monitored using LC-MS and the proteolysis seemed to be determined mainly by the amino acid sequence of β-casein.

Using LC-MS/MS, De Noni (2008) investigated the release of BCM5 and BCM7 during SGID of bovine β-casein variants (A^1, A^2 and B) using pepsin digestion at pH 2.0, 3.0 and 4.0 followed by hydrolysis with Corolase PP^{TM}. β-CN variants were extracted from unprocessed milks and digested after dispersion in bovine milk UF permeate. BCM7 was not released during initial peptic digestion of any of the β-CN variants studied. Regardless of the pH value, B variants of β-casein released during SGID the highest amount of BCM7 (5–176 mmol/mol casein), followed by the A^1 variant. BCM7 was not released from variant A^2 during any steps of SGID process. Furthermore, BCM5 was not formed in hydrolysates irrespective of the genetic variant of the original β-casein or the pH value employed during SGID.

The study of Cieslinska *et al.* (2007) reported the release of BCM7 from a homozygous A^2 milk following digestion (24 h at pH 2.0) with pepsin. Recognition/quantification of BCM7 was done by means of HPLC/UV and the reported release of BCM7 was four times lower from homozygous A^2 milk than from homozygous A^1 milk. However, the exact levels of BCM7 in these milk samples are unknown since the reported data referred to milk extracts only.

Very little information is available on the release of BCMs during digestion of β-casein from goat, sheep or buffalo milk. Petrilli *et al.* (1984) digested β-casein from buffalo milk using pepsin, trypsin, chymotrypsin, elastase, carboxypeptidase A, carboxypeptidase B and leucine amino peptidase (LAP). The digests were analysed chromatographically by HPLC. Neither gastric nor pancreatic enzymes were found to release BCM7. The digests contained a pro-BCM7 with amino acid sequence corresponding to residues 59-68 of buffalo β-casein. However, incubation of this peptide with rabbit small intestinal brush border enzymes did not release BCM7. Petrilli *et al.* (1987) investigated the degradation of a β-casomorphin-containing fragment (tryptic peptide corresponding to residues 49-68 of buffalo β-casein) using pig pancreatic juice. Using fast atom bombardment MS to identify the fragments produced, it was reported that incubation with pancreatic juice was not capable of releasing β-casomorphin or morphiceptin from the tryptic peptide.

Recently, De Noni (2008) did not find BCM5 or BCM7 in the peptic (pH 2.0 or 3.5) digests of milk-based infant formulas following SGID. These products contained both A^1 and A^2

variants of β-casein and it was only upon further digestion with Corolase PP™ that BCM7 levels ranging from 0.02 to 0.37 nmol/mL were detectable.

BCM7 was recovered but not quantified in the UF permeate of a reconstituted infant formula subjected to peptic hydrolysis at pH 3.5 followed by digestion with Corolase PP™ (Hernandez-Ledesma *et al.* 2004). In another study, the same authors did not find the presence of this peptide in an infant formula digested with pepsin (pH 3.5) and porcine pancreatin (Hernandez-Ledesma *et al.*, 2007). However, the peptide (Val^{59})-BCM9 was recovered in the same infant formula. Using HPLC separation and UV detection, Jarmolowska *et al.* (2007b) reported the presence of BCM5 in peptide extracts of an infant formula and in its pepsin–trypsin hydrolysate. The amounts of BCM5 in the infant formula extracts and its hydrolysate were 0.39 and 43.11 μg/mL (0.67 and 74.46 nmol/mL), respectively. No BCM7 was found in the extracts either prior to or after SGID.

3.2.2 Non-milk derived opioids released in SGID

Dietary opioid peptides are present in a variety of protein sources, such as wheat, barley, soy, spinach (rubisco protein) and blood (Table 2). Fukudome and Yoshikawa (1992) found gluten exorphins A5, A4, B4 and B5 in an *in vitro* digest of wheat gluten with pepsin and thermolysin. The same peptides were not released after pepsin digestion alone or in pepsin/trypsin/chymotrypsin digests. However, gluten exorphins were found after further hydrolysis of the peptic digest with microbial neutral proteinases derived from *Aspergillus oryzae* or *Bacillus subtilis.* These results suggested that the *in vivo* release of gluten exorphins A and B from wheat gluten might be accomplished by the concerted actions of pepsin and enterobacterial proteinases. Fukudome and Yoshikawa (1993) isolated the opioid peptide exorphin C from a pepsin-trypsin-chymotrypsin digest of wheat gluten. A subsequent study by Fukudome *et al.* (1997) reported the release of gluten exorphins A and B by the action of pepsin and pancreatic elastase. These exorphins could therefore potentially be released *in vivo* by the action of gastrointestinal proteinases following the ingestion of wheat gluten.

Opioid peptides can also be derived from soy proteins. Agui *et al.* (2006) reported the release of soymorphin-5 after digestion of soy β-conglycinin with pancreatic elastase and LAP. The β-subunit of soy β-conglycinin also contains the sequence (YPWT), similar to human BCM4. Ohinata *et al.* (2007) reported the release of human BCM4 and soymorphin-5 during digestion of a model peptide corresponding to the β-subunit of soy β-conglycinin with pancreatic elastase and LAP.

Bioactive peptides can be generated in the course of catabolic degradation of technofunctional proteins, as reported for hemorphins, a specific group of peptides derived from blood haemoglobin. Hemorphins consist of a family of opioid receptor-binding peptides from 4 to 10 amino acids that are released by proteolytic processing of haemoglobin β-chain (Fruitier-Arnaudin *et al.*, 2005). The first two haemoglobin-derived peptides identified *in vitro* and termed hemorphin-4 and hemorphin-5 were obtained by incubation of bovine blood with gastrointestinal enzymes (Brantl *et al.*, 1986b). These amino acid sequences corresponded to the 34-37 and 34-38 fragments of the β-chain of bovine haemoglobin and 35-38 and 35-39 fragments of the β-chain of human haemoglobin. Two other hemorphins corresponding to fragments 31-40 (LVV-hemorphin-7) and 32-40 (VV-hemorphin-7) of the β-chain of bovine

haemoglobin have been isolated *in vivo* from tissues and from fluids. These two opioid peptides were also generated during *in vitro* pepsin digestion of bovine haemoglobin (Garreau *et al.*, 1995). Jinsmaa and Yoshikawa (2002) isolated hemorphin-5 from human haemoglobin digested with pancreatic elastase. V-hemorphin-5 was also released under the same conditions.

4 POSSIBLE MOLECULAR INTERACTIONS, GENERAL BIOLOGICAL EFFECTS AND FATE OF FOOD-DERIVED PEPTIDES

4.1 Overview

As has become clear from the previous sections, proteins in food products contain peptides which may provoke responses that go beyond typical nutritional or metabolic functions. In some of these cases, direct interaction with an enzyme or with one or more receptors may be involved. Examples include the inhibition of Angiotensin Converting Enzyme (ACE) or the binding to opioid- or other receptors. In other situations, peptides may act in a more indirect way, for example via the immune system or by binding of reactive intermediates formed in biological processes. This chapter discusses some of the actions and mechanisms that have been reported for food-derived peptides, in particular those from milk protein. Some of these peptides have been called exomorphins, due to their interaction with opioid receptors. Exomorphins can be formed from plant- or animal derived proteins, including milk. It is important to note that the fact that these peptides are called exomorphins does not imply that their reported effects can always be explained from interactions with opioid receptors. As will also become clear from this chapter, particular pharmacological or toxicological mechanisms need solid experimental evidence. Moreover, data from *in vitro* studies or studies performed in specific animal models still need to be carefully confirmed by observations in humans, since food derived peptides showing activity *in vitro* might not reach the site of action in the organism when administered via the GI tract and results obtained in animal models might not be transferable to humans.

In order to act systemically, peptides should be absorbed from the GI tract into the circulation in an active form. This implies that the molecules must first resist hydrolysis by brush border peptidases and then be absorbed across the intestinal epithelium. Further degradation can occur by peptidases in the liver, in the bloodstream and in other tissues. A prerequisite to act in the CNS is their ability to pass the blood brain barrier (BBB). Peptidases in the brush-border membrane are actively involved in limiting the absorption of small peptides across the intestinal mucosa. Brush-border peptidases are mainly active against tri-, tetra-, and higher peptides (up to ten amino acid residues), while intracellular peptidases are active predominantly against dipeptides (Pauletti *et al.*, 1996). The amount of peptide which is available to pass into the brain depends on the distribution of the peptide within the blood compartments and its elimination half-life. The presence of peptidases and proteinases in the blood has been shown to affect the bioavailability of many peptides. These enzymes include dipeptidyl(amino) peptidase IV, aminopeptidase A and N, ACE and carboxypeptidase (Egleton and Davis, 1997). In order to act in the CNS, (food derived) peptides must cross the BBB. The BBB will in principle limit the delivery of peptides to the brain. The endothelial

cells that form the BBB are interconnected by tight cellular junctions which provide a high transendothelial electrical resistance. Moreover, the BBB acts as a metabolic barrier equipped with a number of proteolytic enzymes, such as aminopeptidase A, aminopeptidase M, and ACE which are known to degrade peptides (Egleton and Davis, 2005).

4.2 Interactions of food-derived peptides with opioid receptors

4.2.1 The opioid system

Opioid receptors derive their name from the classical effects of the active ingredients of opium, in particular morphine and its derivatives. Following the discovery of the first opioid receptor in 1973 (Pert and Snyder, 1973; Simon *et al.*, 1973; Terenius, 1973) the first two endogenous ligands were quickly identified in bovine brain (Hughes *et al.*, 1975). Since then, several other opioid receptor subtypes have been found, as well as a number of endogenous and exogenous ligands. Opioid receptors belong to the large family of transmembrane-spanning G protein-coupled receptors (GPCRs). Different cellular processes can be linked to opioid receptor activation, including adenyl cyclase, Ca^{2+}-channels, K^{+}-channels, or phospholipase C turnover (Waldhoer *et al.*, 2004). Opioid receptors are most abundant in the central nervous system, but can also be found in peripheral nervous, endocrine and immune tissues, including the intestinal tract (Wittert *et al.*, 1996; IUPHAR, 2008). Opioid receptors are now divided into three different sub-classes. The most current (IUPHAR) recommended nomenclature is to denote these as μ (also called MOP), κ (also called KOP) and δ (also called DOP). There is also a fourth opioid-like receptor, which is now called N/OFQ (nociceptin/orphanin FQ). This receptor is considered 'opioid-related' rather than opioid. It exhibits a high degree of structural homology with the conventional opioid receptors but displays a distinct pharmacology (Alexander *et al.*, 2008) All four receptor groups are about 65% identical to each other with a common three-dimensional structure that spans the cell membrane seven times, forming three extracellular loops and three intracellular loops. The seven helical domains have common features defining an opioid-binding pocket and more divergent aspects that delineate high affinity and selectivity for receptor sub-type specific ligands (Quock *et al.*, 1999). Opioid receptor activation by endogenous and exogenous ligands can result in a multitude of effects which include analgesia, respiratory depression, euphoria, feeding, the release of hormones, inhibition of gastrointestinal transit, and effects on anxiety. In general, agonists selective for μ- and δ-receptors are analgesic and rewarding, whereas agonists for κ are dysphoric (Waldhoer *et al.*, 2004).

Tolerance to opioid ligands is a well-known phenomenon that occurs after chronic exposure to such compounds. On theoretical grounds this could become relevant if food-derived opioid peptides would be present in significant amounts at the receptor binding site during prolonged periods. However, such phenomena have never been described in relation to food-derived opioid peptides. Surprisingly, the mechanism (or mechanisms) underlying the development of for example morphine tolerance has not been completely elucidated (Waldhoer *et al.*, 2004; Bailey *et al.*, 2006; Christie, 2008). Available data suggest that it is unlikely that receptor down regulation is solely responsible for the development of morphine tolerance. Other processes, including desensitization or uncoupling are probably also involved.

4.2.2 Endogenous opioids

Several endogenous ligands for opioid receptors have been discovered. So far, all of these were found to possess a peptide structure. The endogenous ligands are often divided into three groups: endorphins, enkephalins, and dynorphins (Waldhoer *et al.*, 2004; Foord *et al.*, 2005; Fichna *et al.*, 2007; IUPHAR, 2008). Many endogenous opioid peptides are derived from inactive precursor polypeptides, including proopiomelanocortin, proenkephalin, and prodynorphin, respectively (Waldhoer *et al.*, 2004). Several endogenous ligands contain a similar pentapeptide sequence Tyr-Gly-Gly-Phe-Met/Leu (YGGFM/L). Nociceptin/orphanin FQ, however, contains a phenylalanine (F) instead of the N-terminal tyrosine. Endomorphin-1 (Tyr-Pro-Trp-Phe-NH2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH2) are two endogenous tetrapeptides with high affinity and remarkable selectivity for the μ-opioid receptor. See Waldhoer *et al.* (2004) and Balázs (2007) for recent reviews. Other peptides also show some selectivity. Enkephalins act in particular on δ-receptors, dynorphins on κ-receptors, but β-endorphin act both on μ- and δ-receptors with low to moderate affinity only. Endogenous opioid peptides function as inhibitory neurotransmitters and neurohormones. They are released from nerve cells to act on other cells that bear opioid receptors and thus dampen the activity of those cells. They can all modulate the intensity of pain despite the fact that they act through different classes of opiate receptors. Opioids are also involved in complex behaviours, such as sexual attraction, aggressive/submissive behaviours and eating behaviour. They have also been implicated in psychiatric disorders, such as schizophrenia and autism, although the evidence for this is debated. Endomorphins have been detected in significant levels in immune cells and there is persuasive evidence emerging that they can also exert potent anti-inflammatory effects in both acute and chronic peripheral inflammation (Jessop, 2006).

4.2.3 Exogenous opioids

Following the discovery of the medicinal compound morphine several compounds have subsequently been synthesized for medical use. These include very potent drugs like fentanyl, methadone and pentazocine. Among the medically used opioids, there are, so far, no peptides or related compounds. However, it has already been known for almost 30 years that several proteins present in our diet contain sequences that, after being released, can at least theoretically interact with opioid receptors. The discovery of such exogenous opioid ligands was first reported around 1979 (Zioudrou *et al.*, 1979). Since then, several food-derived opioid peptides have been described (see Chapter 2.2). The first pharmacological descriptions of milk-derived peptides that where later called β-casomorphins date back to the late 1970's (Brantl *et al.*, 1979; Zioudrou *et al.*, 1979; Brantl *et al.*, 1981; Brantl *et al.*, 1982; Koch *et al.*, 1985). Using standard pharmacological bioassays, including the guinea-pig ileum longitudinal muscle myenteric plexus preparation (GPI) and the mouse vas deferens (MVD) as well as ligand-binding assays, it was concluded that both bovine and human BCMs predominantly bind to the μ-type receptor. In addition, there seems to be some affinity for the δ-receptors, in particular of human BCM4 and BCM5 (Koch *et al.*, 1985). In the GPI assay, human BCMs (Tyr-Pro-Phe-Val-Glu-Pro-Ile) were found to be 3 to 30 times less potent than the bovine BCMs (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) and 300-600 times less potent than normorphin (Koch *et al.*, 1985). The rank order of potencies for human β-casomorphins was the same as for the

bovine β-casomorphins (BCM5 > BCM4 > BCM8 > BCM7). Brantl *et al.* (1981) estimated that the affinities of the (bovine) β-casomorphins for opiate receptors from rat brain, as indicated by their IC50 values, where a factor > 300 lower than that of morphine. BCM5 was found to be the most potent and BCM7 the least potent.

Compared to milk-derived opioid peptides, the pharmacology of dietary exorphins from other food sources has been studied less well. However, quite a few examples exist. Oryzatensin, a peptide from rice albumin, was shown to possess ileum contracting and immunomodulatory activities (Takahashi *et al.* 1994) along with C3a agonist activity leading to anti-analgesic and anti-amnesic effects after intra-cerebro-ventricular (i.c.v.) administration (Jinsmaa *et al.* 2001, Takahashi *et al.* 1996). Rubiscolin-6 is a peptide formed from the enzyme Rubisco, widely present in green leaves. It was shown to have analgesic activity (Yang *et al.* 2001), to exert stimulated memory consolidation (Yang *et al.* 2003) and to have anxiolytic activities in the elevated plus maze test in mice (Hirata *et al.* 2007). Various blood proteins, including albumin and γ-globulins (Zioudrou *et al.* 1979), and haemoglobin (Brantl *et al.* 1986) can release peptides that act as opioid receptor ligands. Peptides from human haemoglobin β-chain have been related to many *in vivo* physiological effects (coronaro-constrictory, anti-tumor, immunoregulatory activities) and several of the hemorphins interact at various levels of the renin-angiotensin system (RAS) by inhibiting ACE, aminopeptidase N and dipeptidyl peptidase IV activities (Fruitier-Arnaudin *et al.*, 2005). In addition, some hemorphins and in particular LVV-Hemorphin-7 (LVVYPWTQRF), binds with high affinity to the brain (IC50 = 4.15nm) and renal AT4 angiotensin receptor subtype and is possibly the main endogenous ligand from this receptor. A peptide with opioid activity can also be formed from egg albumin (Zioudrou *et al.* 1979). Other studies concerned the presence of opioid peptides in enzymatic digests of wheat gluten. Zioudrou *et al.* (1979) found opioid activity in the peptic digest of wheat gluten. This activity related to both the inhibition of the contraction of the electrically stimulated mouse vas deferens and the inhibition of adenylate cyclase of neuroblastoma X-glioma hybrid cells. Gluten exorphin B5 has been shown to stimulate prolactin secretion in rat (Fanciulli *et al.* 2005). Gliadin peptides were shown to block leucocytes migration which was inhibited in the presence of naloxone (Graf *et al.* 1987). Takahashi *et al.* (2000) studied the effects of gluten exorphin A5 on the pain-inhibitory system, emotionality and learning/memory processes in ddY mice. Thus, exorphin A5 has been found to produce various effects in both the peripheral and central nervous system. According to Sun and Cade (2003), following administration, gliadorphin-7 gains access to brain cells of normal rats by diffusion through circumventricular organs. A sequence identical to human BCM4 (Tyr-Pro-Phe-Val) can be released from the β-conglycinin fraction of soy. Recently, Ohinata *et al.* (2007) described the actions of what they call soymorphins-4,-5,-6 and -7. Soymorphins were tested by the GPI and MVD assay, as well as *in vivo* in mice. They were found to be more potent than human BCMs and showed anxiolytic activity (in the elevated plus-maze test) after oral administration. The authors also simulated enzymatic conditions in which these soymorphins could be formed in the GI tract. In contrast to β-casomorphins (human and bovine), hemorphin and "soymorphins', which show μ-receptor preference, gluten exorphins and rubiscolin are reported to be more δ-receptor specific.

4.3 Non opioid receptor-mediated action of milk-derived peptides

4.3.1 Milk protein-derived immunomodulatory peptides

It has been demonstrated that κ-casein and its caseinomacropeptide 106-169 fragment inhibit both the mitogen-induced Peyer's patch B lymphocytes' proliferation *in vitro* in mice and rabbits. It also inhibits the antibody response induced by sheep red blood cells on mice splenocytes (Otani *et al.*, 1992, 1995a,b,c). In addition, α_{s1}-casein, β-casein and κ-casein hydrolysates inhibit *in vitro* B mitogen (LPS, pokeweed mitogen) or T mitogen (PHA, ConA) induced splenocytes and Peyer's patch lymphocyte proliferation (Otani *et al.*, 1993, 1995 a, b, c, 1996). A group of casein-derived peptides modulate the activity of macrophages, that is B and T lymphocytes (Parker *et al.*, 1984; Berthou *et al.*, 1987; Migliore Samour and Jollès, 1988; Kayser and Meisel, 1996). β-casomorphins may also affect the human mucosal immune system, possibly via opiate receptors in lamina propria lymphocytes (Elitsur *et al.*, 1991).

Two peptides isolated from human β-casein, the hexapeptide 54-59 (Val-Gly-Pro-Ile-Pro-Tyr) and the 60-62 tripeptide (Gly-Leu-Phe), were found to stimulate the *in vitro* phagocytic activity of sheep red blood cells by murine peritoneal macrophages. *In vivo* they prevented an infection of mice following intravenous injection of bacteria (*Klebsiella pneumoniae*). In addition, the opioid peptide isolated from β-casein (residues 63-68) has been shown to stimulate the immune system and particularly phagocytosis. Isracidin, the 1-23 N-terminal fragment from α_{s1}-casein B protects mice against infection by *Staphylococcus aureus* and *Candida albicans* by stimulating phagocytosis and immune response (Lahov and Regelson, 1996). In addition, α-lactalbumin and derived peptides could modulate intestinal cell proliferation and maturation (Alston-Mills *et al.*, 1997). An antimutagenic activity has also been observed in whey protein hydrolysates fermented by LAB (Ganjam *et al.*, 1997; Matar *et al.*, 1997). Furthermore, immunoactive peptides have been produced from α-lactalbumin and lactoferrin. The mechanism by which these peptides exert their immunomodulatory effect has not yet been defined but it may be related to their binding/interaction with opiate receptors.

4.3.2 Other activities of food-derived opioid peptides

A link between hydrolysed casein formulas, BCM7 and α-casein with pseudo-allergic skin reactions in children has been suggested (Kurek *et al.*, 1995; Kurek and Malaczynska, 1999). This observation was based on results from skin prick tests. The skin reactions could be prevented by pretreatment with antihistaminic drugs. In later studies with isolated rat mast cells, α-casein was found to develop a stronger effect than BCM7. The stimulation of opioid receptors (in particular of the μ-type) has been shown to cause pruritus and other sensitivity reactions which can be blocked by anti-histaminics (Ganesh and Maxwell, 2007; Woodall *et al.*, 2008). However, the reports by Kurek may also be related to non-specific mechanisms and do not seem to indicate a specific effect of BCMs.

Ingestion of milk protein components has been linked to tissue development (Britton and Kastin, 1991). For instance, casein-derived peptides have been shown to stimulate mitosis of different cell types (Azuma *et al.*, 1989; Nagaune *et al.*, 1989; Coste *et al.*, 1992) or to affect protein synthesis, proteolysis and ureogenesis (Takenaka *et al.*, 1991). This seems particularly important for immunocompetent cells since milk contains components which can modulate

the non-specific or specific immune systems. Moreover, it has been suggested that milk proteins may be involved in the induction of the autoimmune response in Type-1 or insulin-dependent diabetes (IDDM). This will be discussed in Chapter 4.5.4.

There have been some studies on interactions of casomorphins with receptors other than opioids. For example, Rüthrich *et al.* (1993) suggested that there may be an interaction with dopaminergic regulation systems. This was based on the effects of BCMs on apomorphine-induced hyperlocomotion in rats. However, there are no recent studies to confirm this hypothesis. Recently, one research group has suggested a possible interaction of BCM7 with serotonin systems (Sokolov *et al.*, 2005, 2006, 2008).

4.4 Absorption and fate of peptides

4.4.1 Factors affecting absorption of opioid peptides from the GI tract

The composition of the intestinal content, including food, is subject to significant variation. Gastric emptying and intestinal transit can significantly modify the time that a peptide is present at a site along the GI tract thereby impacting absorption. In addition, and depending on the pKa of the peptide, the pH microclimate could inhibit or favor peptide transport.

In healthy adults the absorption of peptides larger than di-tripeptides is usually highly restricted. In specific situations, such as during stress, certain diseases or aggression, both animals and humans display increased intestinal permeability. In the human neonate, the question as to whether a given peptide can be absorbed is more complex (Vaarala *et al.* 1998, 2008). The neonatal gut is known to be relatively permeable to proteins but with increasing age the permeability of the intestine to proteins and macromolecules decreases. Neonatal permeability of the GI tract for macromolecules and its alteration with time is species-dependent. In piglets, a model close to the human newborn, the transport capacity decreases rapidly within the first days after birth (Pacha *et al.*, 2000). There is a close relation between the degree of maturation and absorptive functions of the intestine. The transport of proteins plays an important function during early postnatal life as it facilitates the absorption of growth factors and immunoglobulins present in maternal milk. This is crucial for ungulates, such as piglets or calves that are born nearly agammaglobulinemic. Nevertheless, other mammals which are born more or less hypoglobulinemic, such as rat, mouse, or man, also receive IgG passively from the maternal milk through absorption in the proximal small intestine. Although intestinal absorption may occur in adults, for example during stress situations (Kuge *et al.*, 2006) this group is less vulnerable and generally already exposed to a much greater diversity of peptides and proteins.

4.4.2 Transfer mechanisms across the intestinal epithelium

There are two distinct pathways for peptides to cross the intestinal epithelium, i.e. the transcellular and the paracellular pathway, respectively. When transported transcellularly, peptides may be metabolised, they can be subject to carrier mediated transport (uptake, efflux, antiport, symport) or they can be transported by transcytosis and/or endocytosis (with or without hydrolysis). The translocation of peptides across Peyer's patches could provide another mechanism of transfer (Des Rieux *et al.*, 2006, Foltz *et al.*, 2008). Opioid peptides are

reported not to be transported by translocation across cellular membranes, that is a mechanism used by cell-penetrating peptides (CPPs) (Patel *et al.*, 2007). The physicochemical properties of peptides, such as molecular size, hydrogen bonding, conformation, partition coefficient, hydrophilicity, lipophilicity, and electrostatic potential can modify the permeation of peptides. Opioid peptides are generally highly hydrophobic. Therefore, their transfer across the intestinal epithelium may not occur easily (via diffusion or paracellular pathways enhanced depending on their hydrophilicity, Iwan *et al.*, 2008). The potential mechanisms of active transfer of opioid peptides across the intestinal epithelium have not been clearly established. The PEPT1, H^+-coupled transporters, are widely expressed in intestinal epithelium and could carry only di- and tri-peptides (Ganapathy *et al.*, 2005). The expression of OATP (Organic Anion Transporting Polypeptides) is widespread (intestine, brain notably) and three synthetic opioids have been shown to be carried by this type of transporter. A new Na^+-dependent active transport (ARPE-19) has been found in a cell line of epithelial retinal pigment (RPE). This transport system accepts a variety of opioid peptides containing 4 to 13 amino acids, the distribution pattern of this transport system in tissues other than RPE in mammals is still unknown (Hu *et al.*, 2003). Regardless of the barriers and the potential transport mechanisms described here it has been recognized that the intestinal mucosa does not present an absolute barrier that totally prevents peptide permeation. Various examples of biologically active peptides, including fragment of α_{s1}-casein (residues 1-23, Chabance *et al.*, 1998) hexarelin (Roumi *et al.*, 2001), pentapeptide from β-casein (HLPLP, Quiros *et al.*, 2008), tetrapeptide (GGYR, Shimizu *et al.*, 1997) octreotide (synthetic octapeptide analog of somatostatin, Dorkoosh *et al.*, 2004) have been reported to cross the intestinal epithelium (using *in vitro* or *in vivo* models). Nevertheless, in all reports to date, peptides and peptidomimetics typically appear to show poor and variable oral bioavailability (Pauletti *et al.*, 1996).

4.4.3 Absorption of opioid peptides

The estimation of peptide bioavailability in humans is generally based on *in vitro* systems or *in vivo* animal models. *In vitro* models which are frequently used to screen for intestinal absorption include monolayers of the human intestinal cell line Caco-2 and freshly excised rat (or other species) intestinal mucosa mounted in Ussing- type chambers. Föger *et al.* (2008) showed that *in vitro* and *in vivo* correlations favour the use of Ussing chambers to predict bioavailability. The Caco-2 cell line, originally derived from a moderately well-differentiated human colon adenocarcinoma (Fogh *et al.*, 1977) was shown to undergo spontaneous *in vitro* enterocytic differentiation (Pinto *et al.*, 1983) leading to the formation of a monolayer of highly polarised cells in 2–3 weeks. These cells are joined by functional tight junctions, with well-developed and organized microvilli on the apical membrane. Differentiation of Caco-2 cells results in the polarised expression of brush border hydrolases (i.e. disaccharidases and peptidases) and of several transport proteins, normally expressed in the absorptive enterocyte of the small intestine (Zweibaum *et al.*, 1991; Hidalgo and Li, 1996). The lack of mucus producing goblet cells is one of the major disadvantages of all cell based *in vitro* models (Hilgendorf *et al.* 2000). Furthermore a practical problem is that permeability data obtained in different laboratories are difficult to compare due to variation in cell line differentiation and selection of sub-populations (Hayeshi *et al.* 2008).

The Ussing chambers technique uses intestinal tissue which is mounted between two half-chambers, establishing a luminal and a serosal side. This allows different compartments of the gastro-intestinal tract, from the stomach to the rectum, to be studied at the same time. The ability to maintain intestinal tissue alive in a controlled environment has brought scientists to use this technique to study the permeability of intestinal tissue to various molecules, including ions, nutrients, drugs, small molecule probes and macromolecules (Boudry, 2005)

Shimizu *et al.* (1997) demonstrated transepithelial transport of (bovine) BCM5 by human intestinal Caco-2 cells. The relative flux of BCM5 increased by treating the layer with an inhibitor of dipeptidyl peptidase IV which is implicated in the hydrolysis of the peptide. Similar results were found by Iwan *et al.* (2008) who showed a significant transfer of BCM7 by Caco-2 cells. The permeability coefficients from apical to basolateral and from basolateral to apical side were not significantly different suggesting passive transport across the Caco-2 monolayer.

In the study of Singh *et al.* (1989), the level of BCM7 immunoreactive material (BCMIR) was compared in plasma of 2- and 4-week old pups and adult dogs after intake of bovine casein-based formula, canine milk and soy protein-based formula. Feeding with bovine casein-based formula increased BCMIR in plasma in 2- and 4-week old pups but not in adult dogs. BCMIR was not found after feeding of soy protein milk. The authors speculated that the intestinal mucosa of the newborn is more permeable to the relatively large peptides due to their immature tight junction through which peptides cross, thereby escaping hydrolysis. According to Singh *et al.* (1989), the BCMIR is longer than the heptapeptide (BCM7) molecule and probably consists of 12-13 amino-acid residues. It is interesting to mention that in this study, feeding with canine milk also increased BCMIR in the plasma of pups. In the same study, Singh *et al.* (1989) showed that BCM7 added to plasma was rapidly degraded whereas the BCMIR was stable in plasma.

Using rabbit ileum mounted in Ussing chambers Mahé *et al.* (1989a) showed that morphiceptin (β-CM4-NH_2) was degraded by the intestinal mucosa whereas the β-[DAla2,4, Tyr5]-CM5-NH_2 peptide resisted hydrolysis and crossed the epithelium intact. The brush-border peptidases seem to be the limiting step of morphiceptin transfer with dipeptidyl peptidase IV enzyme playing a major role. By pre-treatment of the ileum with an inhibitor of dipeptidyl peptidase IV the transfer of morphiceptin across this epithelium can be increased.

Umbach *et al.* (1985) demonstrated the presence of BCMIR in the plasma of newborn calves after milk intake. This BCMIR was not identical to BCM7. Its elution profile during chromatography suggested that a considerably larger peptide exists containing the amino acid sequence of BCM7. The detected material might represent a precursor of BCM7 which could subsequently be released enzymatically at any site in the organism to elicit opioid effects.

Tomé *et al.* (1987) showed that BCM5, BCM4 and the analogue β-[DAla2,4, Tyr5]-CM5-NH_2 caused a naloxone-reversible reduction in short-circuit current after addition on the serosa side of intestinal mucosa. Only the analogue had an effect after addition on the mucosal side. Contrary to the analogue, no intact passage of BCM5 and BCM4 were observed.

Table 7 summarises the results of studies on the absorption of opioid peptides.

Table 7. Summary of studies on absorption of opioid peptides

Peptides/molecules	Model	Transfer	Ref.s
BCM7 and -5	Caco-2	Yes (Transcellular)	Iwan *et al.*, 2008 Shimizu *et al.*, 1997
β-CM4-NH_2 (morphiceptin) Tyr-D-Ala-Phe-DAla-Tyr-NH_2	Ussing Chambers	No (yes with inhibitor of DPP IV) Yes	Mahé *et al.,* 1989a
Casein-based formula	*In vivo* (dog pup plasma)	Yes (BCM7 immunoreactive material)	Singh *et al.,* 1989
unmodified β-CMs β-[D-$Ala^{2,4}$,Tyr^5]CM-5-NH_2	Ussing Chambers	No Yes (Transepithelial transfer)	Tomé *et al,.* 1987
Milk intake	*In vivo* (New Born calves)	Yes β-CM immunoreactive material precursor)	Umbach *et al.,* 1985

4.4.4 Transport in the blood stream

The issue of peptide transport in the circulation is complex. Blood contains substantial activities of peptidase enzymes. The half-life of certain peptides in plasma is very short, with an order of magnitude of one minute (Gardner, 1998). Angiotensin II degradation occurs even within seconds (Moskowitz, 2002, 2003). Nevertheless, peptides can also be more resistant to hydrolysis in blood, because they may be weakly bound to carrier proteins which can protect them. Noncovalent association with albumin has also been shown to extend the half-life of short lived proteins (Dennis *et al.*, 2002). Transferrin (Tf), an iron-transporting glycoprotein, also appears to be an appropriate candidate as a peptide carrier utilizing transcellular transport due to the high expression of the Tf receptor on both the brain endothelium and gastrointestinal epithelium (Tuma *et al.*, 2003). However, although its half-life is extended, a peptide associated with plasma protein is usually unavailable for binding to the target. To our knowledge, *in vivo* half-lives of BCMs in blood have not been measured.

4.4.5 Transfer across the blood-brain barrier (BBB)

The BBB is a physical and metabolic barrier separating the microenvironment of the central nervous system (CNS) from the peripheral circulation. It is located at the level of cerebral microvessel endothelial cells, which possess morphological and enzymatic properties distinct from those of capillaries from other body sections. Some regions of the CNS do not express the classical BBB capillary endothelial cells, but have microvessels similar to those of the periphery. These areas are adjacent to the ventricles of the brain and are termed the

circumventricular organs (CVOs). CVOs are involved in neurohormonal secretion and monitoring of blood composition, in these regions the capillaries are more permeable to solutes (Egleton and Davis, 1997).

Four different Peptide Transport Systems have been described for the transfer of peptides across the BBB. They are referred to as PTS-1, PTS-2, PTS-3 and PTS-4. (Ganapathy *et al.*, 2005). Among these 4, only PTS-1 recognizes opioid peptides. The substrates of PTS-1 include Tyr-MIF-1, Met-enkephalin, Leu-enkephalin, BCM7, and dynorphin 1-8. (Ganapathy *et al.*, 2005). Turner *et al.* (1998) have shown that dynorphin 1-13 can cross the BBB in normal cat hippocampus, cortex and cerebellum tissue.

In conclusion, the hypothesis that an opioid peptide, present and available in the blood can cross the BBB, seems possible.

4.5 Organ and system-specific action of food-derived peptides

Food-derived peptides, depending on their absorption from the GI tract and their subsequent distribution in the body, can potentially act on different tissues and organs. However, the majority of studies to date are related to their possible effects on the gastrointestinal tract, the CNS and the cardiovascular system. Furthermore, the potential connection with an increased risk of developing IDDM has received considerable attention in the scientific literature. These topics will be addressed in this chapter.

4.5.1 Effects of food-derived peptides on gastrointestinal function

Dietary proteins are known to have regulatory effects on digestive and metabolic processes. They modulate mucosal processes and the release of gastro-intestinal hormones, such as gastrin, cholecystokinin,secretin and gastrin inhibitory peptide with a subsequent regulatory effect on gastro-intestinal motility and on gastric and pancreatic secretions (Hara *et al.*, 1992). These effects are partly mediated by amino acids present in the intestinal lumen and in the intestinal mucosa after their absorption. They could also be associated with the release of specific food protein-derived peptides during digestion which act either on the luminal part of the intestinal mucosa or on other targets after their absorption (Daniel *et al.*, 1990). Casein has been demonstrated to efficiently stimulate intestinal secretion, thus suggesting the presence both in animals and in humans of specific casein-derived peptides acting on gastro-intestinal processes (Daniel *et al.*, 1990; Yvon *et al.*, 1994; Mahé *et al.*, 1995, 1996). Caseinomacropeptide, the κ-casein-derived fragment, has been proposed as one of these casein-derived bioactive peptides (Yvon *et al.*, 1994; Beucher *et al.*, 1994; Perdersen *et al.*, 2000). Food-derived peptides, including casomorphins can also have different effects in the intestinal lumen and the intestinal mucosa. More specifically, BCMs can interact with endogenous opioid systems in the gastrointestinal wall in neonates as well as in adults.

Milk proteins and their peptides have been suggested to have an effect on the gastrointestinal ecosystem (Baldi *et al.*, 2005). For instance, casocidin I, the 165-203 fragment released from α_{S2}-casein by chymosin, inhibits the growth of *Escherichia coli* and *Staphylococcus carnosus* (Zucht *et al.*, 1995). β-casomorphins were also shown to modulate intestinal transport of amino acids (Brandsch *et al.*, 1994). β-casomorphin fragments were also demonstrated to modulate mucus secretion by intestinal mucus producing cells (Claustre *et al.*, 2002;

Trompette *et al.*, 2003; Zoghbi *et al.*, 2006). Orally administered milk protein-derived opioid peptides have been demonstrated to influence postprandial metabolism by stimulating pancreatic polypeptide, insulin and somatostatin secretion (Morley *et al.*, 1983; Schusdziarra *et al.*, 1983a,b; Takahashi *et al.*, 1997; Froetschel, 1996). They have also been suggested to attenuate the suppression of fat intake via enterostatin (White *et al.*, 2000). Furthermore, BCMs have been demonstrated to prolong gastrointestinal transit time (Daniel *et al.*, 1990; Becker *et al.*, 1990; Defilippi *et al.*, 1995; Mihatsch *et al.*, 2005). β-casomorphins could also modulate water and electrolyte absorption and exert an anti-diarrheal action in animals and in humans (Daniel *et al.* 1990). Evidence has accumulated that the enhancement of net water and electrolyte absorption by BCMs in the small intestine is a major component of their anti-diarrheal action which could be mediated via sub-epithelial opioid receptors or through specific luminal binding sites at the brush border membrane (Tomé *et al.*, 1987, 1988; Mansour *et al.*, 1988; Mahé *et al.* 1989b).

4.5.2 Effects of food-derived peptides on the central nervous system

4.5.2.1 Opioid receptors in the CNS

Opioid receptors are widely distributed in the brain and are also found in spinal cord and peripheral sensory and autonomous nerves (Wittert *et al.*, 1996; Peckys and Landwehrmeyer, 1999; IUPHAR, 2008). Within the CNS, opioid receptors are involved in several, and rather diverse, effects and actions. These include analgesia, sedation, euphoria, dysphoria, appetite and eating behaviour, respiratory depression, cough reflexes, nausea and vomiting, and pupillary constriction. The μ-receptor, to which BCMs have been reported to bind preferentially (Brantl *et al.*, 1981; Brantl *et al.*, 1982; Koch *et al.*, 1985) shows high expression levels in the thalamus, caudate putamen, neocortex, nucleus accumbens, amygdala, interpeduncular complex, and inferior and superior colliculi (IUPHAR, 2008). The μ receptors are also present in the superficial layers of the dorsal horn of the spinal cord. A moderate density of μ receptors is found in periaqueductal gray and raphé nuclei. These brain regions have a well-established role in pain and analgesia. Morphine and other opiate analgesics, such as fentanyl belong to the most well-known agonists of the μ-receptors. Other physiological functions regulated by μ receptors include respiratory and cardiovascular functions, intestinal transit, feeding, mood, thermoregulation, hormone secretion and immune functions.

4.5.2.2 Actions of food – derived peptides on CNS functions: data from animal studies

There are quite a few studies on CNS effects of BCMs obtained in experimental animals. Except for the study with the related "soymorphins" (Ohinata *et al.*, 2007), the compounds were administered parenterally in all studies reported in the literature. Brantl *et al.* (1981) and Grecksch *et al.* (1981) were the first to report on analgesic activities of BCMs after intra-cerebro-ventricular (i.c.v.) injection to rats. They found that all four β-casomorphins studied (BCM4,-5,-7 and -8) elicited analgesia which could be completely antagonized by naloxone. Compared to other BCMs, BCM7 showed the slowest onset of action, but its effect also lasted for the longest period (90 min). The authors specifically remarked on the long duration of action which was much longer than that of endogenous peptides (for example enkephalins)

they had tested. Following these initial reports, several studies have confirmed CNS related effects of BCMs after parenteral administration to animals. A summary is given in Table 8.

Table 8. CNS related effects of β-casomorphins after parenteral administration to animals

Compounds	Animal model	Route of administration	Effect	Reference
BCM4,5,7,8	Rat	i.c.v.	Analgesic activity which was antagonised by naloxone	Brantl et al., 1981
BCM5	Rat	i.c.v.	Dose-dependent analgesic activity in two different tests, antagonised by naltrexone	Grecksch *et al.*, 1981
BCM4,5,7	Young Chickens	i.c.v.	BCMs reduced separation induced distress vocalisations (DVs). BCM5 was more potent than either BCM4 or BCM7, with BCM7 having a somewhat longer effect. Effects were partially antagonised by naloxone	Panksepp *et al.*, 1984
BCM5 and semi-synthetic derivatives	Rat	i.c.v. and i.v	Analgesis, BCM5 > 20 times less potent than morphine after i.c.v administration	Matthies *et al.*, 1984
BCM7	Rat (pups)	i.p. (1–100 mg/kg)	BCM7 had no effect on waking. At 100 mg/kg signs of sleep changes. No signs of respiratory depression. Naloxone pretreatment (1 mg/kg IP) reversed the effects of BCM7 on sleep.	Taira *et al.*, 1990
BCM7	Rat	i.p. (1 and 5 mg/kg)	Accelaration of learning of a food-procuring habit in a T-maze. Delayed learning of active avoidance response involving the use of a painful reinforcing stimulus	Maklakova *et al.*, 1995
BCM4,5,7	Rat	i.c.v.	Only BCM7 stimulated food intake of high fat meal. Antagonised by enterostatin and naloxone.	Lin *et al.*, 1998
BCM5	Mouse and Neuroblastoma cells	i.c.v. and i.p.	BCM5 (i.c.v.) dose- dependently induced amnesia in mice .I.p administration of BCM5 (0.1–20 mg/kg) had no significant effect on either spontaneous alternation behavior or passive avoidance response. However, from a series of combination studies it was concluded that i.p. of a low dose (1 mg/kg) of BCM5 improves the disturbance of learning and memory resulting from cholinergic dysfunction through central mediation involving μ1-opioid receptors. β -casomorphins, including BCM7 were found to stimulate neurite outgrowth on neuroblastoma cells.	Sakaguchi *et al.*, 2003a; Sakaguchi *et al.*, 2003 b; Sakaguchi *et al.*, 2006
BCM7, rubiscolin-5 and exorphin C	Rat (pups)	i.p.	Postnatal learning. The three exomorphins improved the development of the conditioned foraging reflex in a complex maze. Only BCM7 had an effect (negative) on passive avoidance conditioning. Investigators' conclusion: exorphins, in particular β-casomorphins, can have significant and long-term effects on the environmental adaptation of young mammals.	Dubynin *et al.*, 2008
BCM7	Rat (newborn)	i.p.	*Ex-vivo* activation (DNA synthesis) of proliferative processes in the myocardium and ectodermal and endodermal epithelium of newborn rats.	Maslennikova *et al.*, 2008
Soymorphins-5 (Tyr-Pro-Phe-Val-Val), -6 (Tyr-Pro-Phe-Val-Val-Asn) and -7 (Tyr-Pro-Phe-Val-Val-Asn-Ala)	Mouse	Oral	Anxiolytic activities after oral administration at doses of 10-30 mg/kg in the elevated plus-maze test in mice. Soymorphins were found to be more potent than human BCMs.	Ohinata *et al.*, 2007

i.c.v. = intra-cerebro-ventricular ; i.p. = intra-peritoneal

4.5.2.3 Possible links of β-casomorphins with CNS-associated disorders or diseases in humans

Given the possible link between BCMs and CNS-associated disorders, much attention has been given to autism and to a lesser extent to the issue of ventilation disorders and sudden infant death syndrome (SIDS).

4.5.2.4 Ventilation disorders, sudden infant death, sleep (and sleep apnea)

The respiratory depressing activity of opioids in general is well-known. In the clinical setting, respiratory depression may limit the use of opioid analgesia. The fundamental drive for respiration is generated in the brainstem and is modulated by factors that include conscious inputs from the cortex, central (brainstem), and peripheral (carotid and aortic bodies) chemoreceptors that sense changes in the chemical constituents of blood (Pattinson, 2008). Opioids depress respiration by a number of mechanisms and neuronal sites of action, and differences between various opioids exist, even within a specific class (e.g. μ-receptor binding compounds). There are also links between opioid use, sleep apnea and sleep disturbances in general (Wang and Teichtahl, 2007). Opioid receptors are located in the same nuclei that are active in sleep regulation and opioid peptides are suggested to be involved in the induction and maintenance of the sleep state. Abnormal sleep architecture has been reported during the process of opioid induction, maintenance and withdrawal. During induction and maintenance of opioid use there is a reduction of rapid eye movement (REM) sleep and slow wave sleep. More recently, central sleep apnea (CSA) has been reported with chronic opioid use and 30% of stable methadone maintenance treatment patients have CSA.

The pathogenesis of SIDS is complex and multifactorial. Sun *et al.* (2003) have reviewed the possible relationship between BCMs and SIDS. This review is based on the assumption that BCMs might be absorbed from the infant's GI tract and that they also easily pass through the BBB because of the infant's immature CNS. Based on these assumptions, the authors speculate that in infants with abnormal respiratory control and vagal nerve development, opioid peptides derived from milk might induce depression of the brain-stem respiratory centers, leading to apnea and death. As bovine BCMs might be more potent than their analogues in human milk or have different relative receptor preference this might increase the risk for SIDS. However, Sun *et al.* (2003) also mention that infants fed either formula preparations or human milk have a similar risk of developing SIDS.

4.5.2.5 Autism

Autistic spectrum disorders (ASDs) usually include autism, atypical autism, Asperger's syndrome and some related disorders (Cass *et al.*, 2006) Autism is a complex neurodevelopmental disorder characterized by impaired reciprocal social interaction, impaired communication, and restricted, repetitive, or stereotyped behaviors (Barbaresi *et al.*, 2006). The population of children with autism is very heterogeneous. Autism and related conditions in the autism spectrum have become the focus of intense interest which is also stimulated by public concerns about the apparent increase in the number of children with these developmental disorders. The aetiology of ASDs is still unclear, and medical or psychological treatment options often provide disappointing results. Probably also as a result of this,

considerable attention is being paid to nutritional, other environmental factors and complementary therapies (Barbaresi *et al.*, 2006).

Among these, the "leaky gut" concept has attracted much attention. This concept is often also linked to another hypothetical cause, which is vaccination. For example, there have been concerns about the potential role of the measles-mumps-rubella (MMR) vaccine in the causation of autism. This theory suggests that the MMR vaccine produces enterocolitis, thereby causing or increasing a "leaky gut". In the meantime, epidemiologic studies have failed to show an association between the MMR vaccine and autism. The "leaky gut" concept itself has been fueled in particular by the publication of Cade *et al.* (2000) which is based on the increase of peptide containing peaks in urine samples of autistic and schizophrenic patients. However, these peaks have only been partly characterized. Moreover, the dietary intervention that apparently led to improvements was both gluten- and casein free. Nevertheless, the "leaky gut" concept has received considerable support and has become the basis of diets that exclude gluten and casein for ASD patients. Further support has come from Reichelt and Knivsberg (2003) who presented an "autism model", suggesting that exorphins and serotonin uptake modulators are key mediators in the development of autism. They speculated that this is due to a genetically based peptidase deficiency in at least two or more peptidases and/or of peptidase regulating proteins.

Since then however, this model has been questioned by many authors. In subsequent studies using more sensitive and specific LC/MS based analytical methods, the peptides could not be detected. Hunter *et al.* (2003) tried to confirm the presence of opioid peptides in the urine of children with autism and to determine whether dipeptidyl peptidase IV is defective in children with autism. However, they were not able to detect opioid peptides nor did they find any proof for a dipeptidyl peptidase IV deficiency in these children. Recently, Cass *et al.* (2008) using MALDI-TOF MS to analyse opioid-derived peptides concluded that there was no evidence for any opioid peptiduria in children with autism. They state that "opioid peptides can neither serve as a biomedical marker for autism nor be employed to predict or monitor response to a casein- and gluten-free diet". Christison and Ivany (2006) reviewed the gluten and casein elimination diets, concluding that the currently available data are inadequate to guide treatment recommendations and that diets eliminating both gluten and casein (rather than either alone) should be studied first and that outcome measures should include assessments of nonverbal cognition. Dettmer *et al.* (2007) were also unable to detect gliadinomorphin, BCMs, deltorphin 1, or deltorphin 2 in urine of children with ASD. A recent Chochrane review (Millward *et al.*, 2008) also concluded that despite the high rates of use of complementary and alternative therapies (CAM) for children with autism including gluten and/or casein exclusion diets, current evidence for the efficacy of these diets is poor. In conclusion, recent data do not provide any support to link intake of casein or peptides derived from it to ASD.

4.5.3 Effect of milk- derived peptides on cardiovascular health

In relation to cardiovascular health most attention has focused on the antihypertensive properties of food-derived peptides. In animal models and human intervention studies, several of these peptides, including those that can be formed from milk proteins, have been shown to

reduce blood pressure. It was recently demonstrated that the lactotripeptide Ile-Pro-Pro selectively escapes intestinal degradation and reaches the circulation undegraded (Foltz et al.2007). The properties and clinical effects of antihypertensive milk peptides have been reviewed in many recent papers (Korhonen and Pihlanto, 2006; FitzGerald and Meisel, 2007; Hartmann and Meisel, 2007; Hong et al., 2008; Möller et al., 2008; Saito, 2008) The most important mechanism, at least *in vitro*, seems to be inhibition of ACE. Although the discussion about its relevance and the long-term effects on clinical endpoints continues, the reduction in blood pressure by food-derived peptides is generally considered as beneficial.

4.5.3.1 Oxidation of LDL

In relation to casomorphins, there have been some suggestions that BCM7 could be pro-atherogenic (Allison and Clarke, 2006) This effect is supposed to be mediated via stimulation of the oxidation of low-density lipoproteins (LDL).

The LDL fraction is an important lipid carrier in plasma, and consists of cholesteryl ester, phospholipids, free cholesterol and triglyceride, and apolipoprotein B100 (ApoB100) (Stocker and Keaney, 2004). LDL is derived in the circulation from VLDL. It is essential for the transport of cholesterol to peripheral tissues and the regulation of cholesterol metabolism at these sites. Like VLDL. LDL contains ApoB100 as apoprotein, a 500 kDa single peptide chain synthesised in the liver. LDL is extremely susceptible to oxidative damage (Steinberg, 1997; Steinberg, 2002; Stocker and Keaney, 2004; Beck *et al.*, 2008; Matsuura *et al.*, 2008).

This oxidation is considered as a key factor in the pathogenesis of atherosclerosis and cardiovascular disease. Elevated circulating oxidised LDL particles (oxLDLs) are associated with more severe atherosclerotic lesions. High circulating levels of oxLDLs have been shown to be independently predictive of sub-clinical atherosclerosis and acute coronary heart disease. It is assumed that LDL oxidation does not take place in the circulation because of the high anti-oxidant activity in plasma. Instead, LDL is supposed to be oxidised in the sub-endothelial space where it may be exposed to cell-derived free radicals and other reactive oxygen- and nitrogen species. The bidirectional transit of LDL across this space may also result in a small amount of circulating oxLDL (Stocker and Keaney, 2004). When LDL is oxidised, the molecule is recognized by scavenger receptors present on macrophages which do not bind native LDL. These macrophages can become lipid laden, transforming to so-called foam cells. In addition, oxLDL has many other properties that are potentially pro-atherogenic. For example, oxLDL is itself directly chemotactic for monocytes and T cells (Steinberg, 2002). Oxidised LDL is immunogenic (Matsuura *et al.*, 2008). A large number of epitopes within the ApoB100 component of oxidised LDL have been identified that provoke an immune response (Stocker and Keaney, 2004). As a result, antibodies against oxLDL and its complexes may also be markers of cardiovascular diseases. It should be noted that in addition to LDL oxidation, other processes, such as inflammation, are important in the development of atherosclerosis (Steinberg, 2002). In addition to foam cells, other cell types, such as vascular smooth muscle cells and lymphocytes participate in plaque formation (Stocker and Keaney, 2004). In LDL oxidation, both the lipids and the apoB100 are oxidised. LDL oxidation is stimulated in a lipid-rich environment and probably also after consuming a

high fat meal which stimulates oxidative stress conditions (Campbell *et al.*, 2006). Furthermore, it is increased by cigarette smoking and with obesity (Beck *et al.*, 2008).

Several anti-oxidants have been shown to inhibit LDL oxidation (Campbell *et al.*, 2006; Lapointe *et al.*, 2006). The *in vivo* evidence is less consistent, especially with studies using flavonoid or vitamin supplements (Lapointe *et al.*, 2006). A healthy food pattern, such as the Mediterranean-style diet has been associated with decreased oxidation of LDL, whereas low serum carotenoids have been associated with higher ox-LDL (Lapointe *et al.*, 2006; Beck *et al.*, 2008).

Other compounds that may inhibit LDL oxidation include estrogens, melatonin and brain monoamines (Lin *et al.*, 2006). Most interestingly, also peptides can act as inhibitors of LDL oxidation. Lin *et al.*, (2006) reported that endomorphin-1 and endomorphin-2 may inhibit the formation of oxLDL. The anti-oxidant properties of small peptides in general, including those of enkephalins have been more extensively studied. Several peptides possess free radical scavenging activities and the capacity to reduce reactive oxygen species (ROS)-induced lipid peroxidation (Coccia *et al.*, 2001; Fontana *et al.*, 2001). Food-derived peptides can also act as anti-oxidants. This includes peptides from dairy sources, including bovine (Suetsuna *et al.*, 2000) and ovine (Gómez-Ruiz *et al.*, 2008) casein and bovine whey (Peng *et al.*, 2008). The original hypothesis of a pro-oxidant effect of BCM7 dates back to the report of Torreilles and Guerin (1995). These authors report the *ex-vivo* stimulation of LDL oxidation by bovine casein hydrolysates with tyrosyl residues. However, Tyr-residues are very common in food-derived peptides. The findings of Torreilles and Guerin are in contrast to later reports and may possibly be due to specific experimental conditions. Apart from the original communication proposing a role for BCM7 in LDL oxidation (Torreilles and Guerin, 1995) there are no other studies reported to confirm this hypothesis.

4.5.3.2 Effects of BCM7 in a rabbit model for atherosclerosis

The publication of Tailford *et al.* (2003) describing the effects of β-casein A^1 versus β-casein A^2 on atherosclerosis development in a rabbit model for restenosis has received considerable attention. In this model endothelial injury is induced using a balloon catheter inserted into the carotid artery (Manderson *et al.*, 1989). Following surgery, rabbits were fed for 6 weeks a diet containing 0, 5, 10 or 20% casein isolate, either β-casein variant A^1 or A^2 made up to 20% milk protein with whey. Some groups had their diets supplemented with 0.5% cholesterol. The authors reported that in the absence of dietary cholesterol, β-casein A^1 produced significantly higher serum cholesterol, LDL, HDL and triglyceride levels than on a whey diet alone which in turn produced higher levels than β-casein A^2. Rabbits fed β-casein A^1 were found to have a higher percent surface area of aorta covered by fatty streaks than those fed β-casein A^2 and the thickness of the fatty streak lesions in the aortic arch was significantly higher. From their results, the authors concluded that β-casein A^1 was atherogenic whereas β-casein A^2 was not. However, according to the journal editor, this conclusion can be considered as premature (Mann and Skeaff, 2003). In the model used, an acute tissue repair process is dominating which is different from the slowly developing atherosclerosis process as it occurs in man. Fatty streaks are made up of foam cells which during normal atherosclerosis develop from macrophages that have taken up oxLDL. In a damaged vessel

wall there may be considerable oxidative stress which induces oxLDL formation. The model have also been found to be sensitive to variations in dietary composition in general (Stocker and Keaney, 2004) and the rabbit is particularly sensitive to cholesterol. Therefore, small differences in dietary composition not related to casein may also have played a role.

Given the specific limitations of the experimental animal model (rabbit restenosis model) the study of Tailford *et al.* (2003) does not appear to be relevant to support the suggested pro-atherosclerotic role of BCM7.

4.5.3.3 Human studies

The hypothesis suggesting a possible health impact of BCMs and related peptides, in particular BCM7, is largely based on ecological studies correlating a few environmental factors and the prevalence/incidence of some non-communicable diseases. General considerations on human correlational studies and additional considerations on their uncertainty are, therefore, appropriate (Morgenstern and Thomas, 1993). The relationship between a disease and genetic, behavioural or environmental factors is complex. The establishment of a mono-causal theory to explain the prevalence/incidence of a disease is therefore problematic. The most appropriate way to confirm a hypothesis is to conduct a prospective cohort study at an individual level throughout life, collect complete data on genetics, diet, lifestyle and environment and analyse the data on the disease prevalence/incidence while adjusting for all other possible influencing factors.

However, due to the large amount of data which has to be collected and the time such a study would take, many other approaches are used. Examples are shorter prospective studies and trials with different endpoints as for example risk factors for the disease in question. For many diseases this is extremely hard as risk factors are not known or not measurable.

Another choice is represented by a case-control study matching to each observed case (person with diagnosed disease) a fixed number of controls (persons without the disease). Other influencing factors, including sex, age, time point, living region and social status should be as much as possible similar between the case and the controls. At the same time the variation in the suspected influencing factor should be large enough to observe an effect, which can be distinguished from a random variation. However, case-control studies need to assess retrospectively the value of the suspected influencing factor, when it is supposed to have caused the disease. This can vary for some diseases from the prenatal period and infancy to adulthood, therefore some important information may not be available. The same problem arises when other influencing factors can be presumed but not assessed. These factors which are unknown but which may have an impact on the disease are called “confounders” or “confounding factors”. These weaknesses justify the fact that case-control studies are less credited to show evidence for an assumed relationship.

An additional way to test a hypothesis is to conduct an ecological study. This approach has intrinsic weakness. Instead of collecting data at individual level, summarised data at group level are used and compared. These data are generally easier to access but the implicit assumption of homogeneity at individual level is in most cases incompatible with a use of the

results of the study to demonstrate a cause – effect association. This can only be a first step to gain evidence for a hypothesis.

The processes leading to major cardiovascular diseases (CVD), such as ischaemic heart disease and stroke, are multifactorial and develop usually over a long period before diagnosed, most typically, after the age of 50 to 60 years. CVD is considered as a lifestyle disease complex and in many countries, in particular in countries with high living standards, the prevalence of CVD is high being the major cause of mortality (WHO, 2003). Therefore, CVD has been extensively investigated for decades. Many risk factors have been defined and some of them develop over a series of years, even from early childhood. These include high blood pressure, high LDL cholesterol, low HDL-cholesterol, inflammatory markers, homocysteine, smoking, physical inactivity and central obesity. On the other hand, the increased prevalence of obesity in young populations has been associated with increased prevalence of type 2 diabetes among young people. Type 2 diabetics have an increased risk of CVD. Current scientific research associates CVD and type 2 diabetes with inflammatory, immunological and metabolic disorders in the organ functions. The fundamental mechanisms of these dysfunctions are not fully understood but recent hypotheses implicate chronic inflammation as one of the causal factors. The potential role of diet in regulating the initiation and development of inflammatory processes in the body has been recently reviewed (Nicklas et al. 2005).

Some ecological studies have linked the intake of β-casein A^1 with cardiovascular disease mortality. These studies have used an estimated per capita consumption of β-casein A^1 from dairy foods, based on other studies and statistical reports. The compiled data included for example protein content and allele frequency distributions of β-casein in milk of different cow breeds in selected countries. The calculated β-casein A^1 intake figures were then correlated to the CVD prevalence in the same countries.

An early report hypothesised an association between estimated milk protein intake (not including cheese) and deaths from coronary heart disease (Seely, 1981). McLachlan (2001) subsequently suggested a strong correlation between the estimated per capita intake of β-casein A^1 (excluding protein from cheese) in 16 countries around 1980 and ischaemic heart disease mortality as recorded 5 or 10 years later. In the same study he suggested that "there may be an intimate relationship between smoking, the consumption of β-casein A^1 and deaths from CVD and lung cancer".

Laugesen and Elliott (2003a,b) also reported a correlation between the calculated per capita consumption of β-casein A^1 (excluding protein from cheese and butter) and the prevalence of ischaemic heart disease mortality in 20 countries. However, in contrast with the previously mentioned study, this latter study could not find a relationship between the consumption of tobacco and CVD. This illustrates the fact that ecological studies cannot adjust for all the confounding factors.

Using data from the 1970s, Hill *et al.* (2002) reported a similar association as the above researchers concerning the consumption of β-casein A^1 and the prevalence of CVD. However, when using data from the 1990s the association was weak. These authors concluded that since the relative proportion of β-casein A^1 was apparently increasing in milk over this time period

in the relevant countries, the consumption of this protein had no effect on ischaemic heart disease related mortality (Hill *et al.* 2002). In contrast to the above studies, more recent large cohort studies have suggested a beneficial role for milk consumption in the prevention of heart disease and stroke (Elwood *et al.* 2004a,b, 2005a,b).

Two human intervention studies have been published about the possible relationship between consumption of β-casein A^1 or A^2 and CVD. Chin-Dusting *et al.* (2006) performed a double blind crossover study in 15 subjects who received 25 g/day of β-casein A^1 compared with the same dose of β-casein A^2 for 12 weeks. No differences were found in any of the endothelial and functional tests performed between the two treatments. Venn *et al.* (2006) tested whether β-casein A^1 and A^2 variants differentially affected plasma cholesterol concentrations in humans. In a randomised crossover trial with 62 subjects of two four-and-a-half week periods without washout they found no evidence that dairy products containing β-casein A^1 or A^2 exerted differential effects ($P > 0.05$) on plasma cholesterol concentrations. According to these results, β-casein A^1 does not seem to be linked to cardiovascular disease. Even though randomised intervention studies are considered to provide the strongest body of evidence the above studies do not allow firm conclusions to be made, given the limited number of subjects and the short intervention periods.

Therefore, overall, it can be concluded that, to date no definitive scientific data exist that link consumption of β-casein variants with increased risk of CVD in humans.

4.5.4 Food-derived peptides and type 1 diabetes mellitus

4.5.4.1 Pathogenesis and incidence of type 1 diabetes

Type 1 diabetes (insulin dependent diabetes mellitus) is an autoimmune disease induced in genetically predisposed individuals by environmental factors and results from the progressive destruction of insulin secreting pancreatic β-cells by autoreactive T-lymphocytes and macrophages, leading to insulin deficiency, probably over a period of months or years (Atkinson and Maclaren, 1994; Simone and Eisenbach, 1996; Rabinovitch and Suarez-Pinzon, 1998; Schranz and Lernmark, 1998). At least half of the patients are diagnosed before or around reaching puberty (Atkinson and Eisenbarth, 2001). The development of type 1 diabetes in genetically susceptible individuals has been divided into a series of steps, including its "triggering" by environmental risk factors, the induction of active cellular autoimmunity against islet cells often associated with the presence of auto-antibodies and the loss of β-cell function and progressive total or near total β-cell destruction with insulin dependence.

Type 1 diabetes is the second most common chronic childhood disease in the world after allergy (David *et al.*, 1994). The incidence has been increasing worldwide although there is large geographical variation (Akerblom and Knip 1998; Onkamo *et al.*, 1999; Atkinson and Eisenbarth 2001; Peter 2007). It has been suggested that the incidence of type 1 diabetes will be about 40% higher in 2010 than in 1998 (Onkamo *et al.*, 1999). The increase in recent years has been most pronounced in very young children (Tuomilehto *et al.*, 1999; Dahlquist and Mustonen 2000; Pozzilli *et al.*, 2007). Incidence figures originate from researchers and registers within a country, sometimes collected in review papers (Onkamo *et al.*, 1999), or

they are collected by organizations, such as the DIAMOND study of the World Health Organization (Karvonen *et al.*, 1993; Karvonen *et al.*, 2000; DIAMOND 2006) or the EURODIAB group (Green *et al.*, 1992; EURODIAB 2000). Incidence figures represent the whole country or specific regions. The highest incidence is found in Caucasian populations and particularly in Northern Europe (Onkamo *et al.*, 1999). Finland is reported to have a very high incidence of type 1 diabetes. This may be due to a special genetic susceptibility to type 1 diabetes and exposure to specific, as yet unknown, environmental factors that trigger the disease more efficiently (Tuomilehto-Wolf *et al.*, 1989; Reijonen *et al.*, 1991).

Type 1 diabetes patients present autoantibodies that recognise insulin secreting pancreatic β-cell protein autoantigens. These autoantibodies are measured and used to identify subjects at risk of developing type 1 diabetes. In 80-90% of patients, autoantibodies can be detected against at least one of three islet cell autoantigen (ICA) proteins, including insulin or proinsulin, the smaller form of the neuroendocrine enzyme glutamic acid decarboxylase (GAD65) and a 40-kDa tryptic fragment of the β-islet cell tyrosine phosphatase (called ICA512 or IA-2) (Ziegler *et al.*, 1991; Julier *et al.*, 1991; Hawa *et al.*, 1997). These autoantibodies are associated with a high risk (68-90%) for development of the disease (Verge *et al.*, 1996; Pietropaolo *et al.*, 1998). Auto-antibodies to other islet cell proteins are less frequent and include GAD67 that cross-reacts with GAD65 and possibly ICA69 (also named p69), a 69kD β-cell membrane protein which is inducible by interferon gamma and carboxypeptidase (Castano, 1991; Pietropaolo *et al.*, 1993; Riechter *et al.*, 1993; Lampasona *et al.*, 1994; Martin *et al.*, 1996). Autoantigen-specific T cell clones have also been described from patients with type 1 diabetes (Naquet, 1988; Hammer *et al.*, 1993; Atkinson *et al.*, 1994; McLaurin *et al.*, 1995; Rammensee *et al.*, 1995; Endl *et al.*, 1996; Wicker *et al.*, 1996; Palmer *et al.*, 1997; Verheijden *et al.*, 1997; Geluk *et al.*, 1998; Schloot *et al.*, 1997). Nevertheless, in contrast to other autoimmune conditions in which the autoantibodies may be directly responsible for disease pathology (Solimena *et al.*, 1990; Blackett *et al.*, 1994; Bhol *et al.*, 1995; Elkon 2000) the role of autoantibodies in type 1 diabetes is currently not known and in no case have autoantibodies been proven to be directly involved either in tissue destruction or in disease progression (Hagopian *et al.*, 1993; Kim *et al.*, 1994; Wucherphennig *et al.*, 1995, 1994; Bach *et al.*, 1997; Patel *et al.*, 1997; Falcone *et al.*, 1998). Studies support the findings that T cell proliferative responses to self peptides are not disease-specific (Cosgrove *et al.*, 1991; Kileen *et al.*, 1993; Fugger *et al.*, 1994; Van der Auwera *et al.*, 1996; Undlien, 1997).

The finding that a gut-specific adhesion molecule (a4b7-integrin) was expressed in a specific population of β-cell-reactive T-lymphocytes was discussed as a link between the gut immune system and type 1 diabetes (Paronen *et al.*, 1997). It was hypothesised that dysregulation of the gut immune system could result in an unspecific stimulation of recirculating lymphocytes which finally react with pancreatic antigens (Akerblom and Vaarala, 1997).

4.5.4.2 Genetic and environmental factors and involvement of milk in type 1 diabetes

The genetic origin of type 1 diabetes is clear (Redondo *et al.*, 1999; Zalloua *et al.*, 2002) but genetic predisposition alone is not sufficient to cause the disease. The prevalence of type 1 diabetes in the general population is low (0.5-1%) while 20-30% of the population have disease-susceptible human leukocyte antigen (HLA) alleles.

The risk of type 1 diabetes is higher in monozygotic twins than in dizygotic twins and first-degree relatives, but the concordance rate for monozygotic twins is only 30-50% (Kyvik *et al.*, 1992; Kaprio *et al.*, 1995). Only 10-15% of all new cases of type 1 diabetes have a close family history of the disease (Tuomilehto *et al.*, 1992; Bingley *et al.*, 1993; Atkinson and Maclaren, 1994; Dahlquist and Mustonen, 2000). The association between autoimmune disease and certain major histo-compatibility complex-HLA class II alleles has been described (Svejgaard *et al.*, 1982). The main locus defining the genetic susceptibility to type 1 diabetes is encoded within the HLA region on human chromosome 6 (Thomson, 1984; Todd *et al.*, 1987; Morel *et al.*, 1988; Schrezenmeier *et al.*, 1993; Davies *et al.*, 1994; Todd and Farrall, 1996; Sanjeevi *et al.*, 1996; She *et al.*, 1996; Buzzetti *et al.*, 1998; Sanjeevi 2000). HLA diversity in different populations may have implications for both diabetes prediction and incidence. Several non-HLA gene associations have also been identified, such as the insulin gene region but their exact role in the risk of type 1 diabetes awaits clarification (Davies *et al.*, 1994; Buzzetti *et al.*, 1998; Ikegami *et al.*, 2006). Other candidate loci associated with this disease have also been proposed on chromosomes 2, 11, and 15 (Field *et al.*, 1994; Hashimoto *et al.*, 1994; Owerbach and Gabbay, 1995; Nistico *et al.*, 1996; Larsen *et al.*, 1999; Marron *et al.*, 2000).

The pathologic development of the disease is the result of a combination of genetic and environmental risk factors (Elliott, 1989; Atkinson and Maclaren, 1994; Dahlquist, 1994; Drash *et al.*, 1994; Akerblom and Knip, 1998; Schrezenmeir and Jagla, 2000). Environmental factors have a high impact on disease development (60% to 70%) and the increasing incidence and prevalence of diabetes worldwide can only be explained by environmental changes (Amos *et al.*, 1997; King *et al.*, 1998). The importance of environmental factors in the aetiology of type 1 diabetes is indicated by the observation that the risk that monozygotic twins both develop diabetes is low (Kaprio *et al.*, 1992; Kyvik *et al.*, 1995), by its different incidences in nations that are genetically close, such as the Nordic countries (Wijsman 1984; Backman *et al.*, 1998) and by migration studies showing that the migrant population often develops towards a similar incidence to that found in the new country (Bodansky *et al.*, 1992; Elliott 1992; David *et al.*, 1994). The possible mechanisms by which environmental risk factors trigger type 1 diabetes are classified in three main non exclusive categories. They include (1) a direct initial inflammatory disruption of pancreatic islets by "toxic agents"; (2) an immunological effect in part ascribed to structural similarities between certain regions of foreign proteins and islet cell antigens; (3) an impairment of both the discrimination between “foreign” and “own” haptens (Leech 1998) and the gut associated immune system (Vaarala, 2008).

Dietary risk factors are important in type 1 diabetes. Some studies have found an association between type 1 diabetes and short duration of breast feeding, early introduction of cow’s milk, consumption of milk in childhood or per capita milk consumption (Borch-Johnsen *et al.*, 1984; Mayer *et al.*, 1988; Siemiatycki *et al.*, 1989; Scott 1990; Dahl-Jorgensen *et al.* 1991; Virtanen *et al.*, 1991; Kostraba *et al.*, 1993; Virtanen *et al.*, 1993; Fava *et al.* 1994; Gerstein 1994; Virtanen *et al.*, 1994a, b, c; Vaarala *et al.*, 1995; Norris and Scott 1996; Perez-Bravo *et al.*, 1996; Gimeno *et al.*, 1997; Virtanen *et al.*, 1998; Schrezenmeir and Jagla, 2000). However, such an association is not observed in all the studies (Nigro *et al.*, 1985; Fort *et al.*,

1986; Kyvik *et al.*, 1992; Samuelsson *et al.*, 1993; Bodington *et al.*, 1994; Patterson *et al.*, 1994; Norris *et al.*, 1996; Meloni *et al.*, 1997; Thorsdottir *et al.*, 2000; Zalloua *et al.*, 2002). In addition, regions with different levels of milk consumption present similar incidence of diabetes (Reunanen *et al.*, 1982; Fava *et al.*, 1994; Muntoni *et al.*, 1997; Elliott *et al.*, 1999; Lien *et al.*, 1999; Thorsdottir *et al.* 2000; Kaminsky, 2007).

Other dietary risk factors include soy milk formulas, gluten and wheat consumption at young age (Scott *et al.*, 1997; Akerblom and Knip 1998; Strotmeyer *et al.*, 2004; Frisk *et al.*, 2008). About one out of 20 type 1 diabetics also has coeliac disease (Savilahti *et al.*, 1986; Atkinson and Eisenbarth 2001; Hansen *et al.* 2001; Franzese *et al.* 2007; Dubois PC and van Heel 2008; Frisk *et al.*, 2008). Moreover, solid food intakes resulted in a higher risk in individuals genetically predisposed to diabetes (Kostraba *et al.*, 1992, 1993; Perez-Bravo *et al.*, 1996). Certain toxic chemicals are also proposed as risk factors. These include N-nitroso derivatives, such as streptozotocin (Knip and Akerblom, 1999), a compound toxic to β-cells and extensively used in inducing type 1 diabetes in experimental animals (Lampeter *et al.*, 1989). A possible role of nitrates has been suggested in both an animal and an observational study (Helgason and Jonasson 1981; Helgason *et al.*, 1982). This finding has been supported by some epidemiological studies (Virtanen *et al.*, 1994b) while some others do not find an association (Muntoni *et al.*, 2006a,b). In contrast, vitamin E (Knekt *et al.*, 1999) and vitamin D (EURODIAB 1999; Stene *et al.*, 2000; Hypponen *et al.*, 2001) are considered potential preventive factors in humans. Supplementation studies ongoing for several years among children at risk for diabetes have investigated the effect of nicotinamide suggesting it to have a beneficial effect (Elliott *et al.*, 1996b; Lampeter *et al.*, 1998) although this has later been disputed (Hypponen, 2004).

Other risk factors are also involved in type 1 diabetes. High growth rate has been proposed as a risk factor (Blom *et al.* 1992; Johansson *et al.*, 1994). It has been discussed whether higher weight gain in infants fed formula compared to breast milk might explain the relationship seen as not being due to the consumption of cow's milk (protein) *per se* (Johansson *et al.*, 1994; Akerblom and Knip, 1998). Accelerated linear growth and increasing body mass has been suggested to contribute to the rising incidence of childhood type 1 diabetes. This effect might be mediated through increased beta-cell stress induced by hyperinsulinemia and decreased insulin sensitivity (Knip *et al.*, 2008). Other proposed risk factors include coffee or tea consumption (Virtanen *et al.*, 1994c), physiological stress (Atkinson and Eisenbarth, 2001), climatic variations (Helgason *et al.*, 1992), exposure to insecticides (Akerblom and Knip, 1998), poor sanitation, lack of access to health care and vaccination (Atkinson and Eisenbarth, 2001). Lastly viral infections, such as coxsackie B4 virus and the cytomegalovirus are probably also involved (Akerblom and Knip, 1998; Dahlquist, 1997). Coxsackie B4 virus is one of the viruses most associated with type 1 diabetes and some studies show the presence of immune responses to coxsackie virus antigens in type 1 diabetes children and suggest its implication with type 1 diabetes autoimmunity. This may be either by direct inflammatory disruption of islets or induction of an immune response (Juhela *et al.*, 2000; Serreze *et al.*, 2000; Jaïdane and Hober, 2008). In contrast, a reduced risk has been associated with multiple infections during the first few years of life, while an increased risk has been associated with perinatal infections. A protective effect of preschool day care has been suggested. This theory

has been linked to the age-dependent modifying influence of infections on the developing immune system (Atkinson and Eisenbarth, 2001).

4.5.4.3 Specific immune response to cows' milk protein in type 1 diabetes

The enhanced cellular and humoral immune responses to dietary antigens observed in type 1 diabetes have not been directly implicated in the pathogenetic process. Alternatively, it could be explained by an impaired tolerance to dietary antigens due to regulatory defects in the gut immune system in general (Brandtzaeg, 2002; Vaarala, 2002; Flohé *et al.*, 2003). Given the strong association between the HLA class II genes and susceptibility to autoimmune disease, the complex between an autoantigenic peptide, an MHC (Major Histocompatibility Complex) molecule and a T cell receptor is an obvious focus of interest (Parry *et al.*, 1998). Several studies have investigated antibody as well as T cell responses to distinct cow's milk proteins in diabetics compared to controls to identify possible diabetogenic factors. The main suspected milk antigens are bovine serum albumin (BSA), bovine insulin, β-lactoglobulin and β-casein (Karjalainen *et al.*, 1992; Drash *et al.*, 1994; Vaarala *et al.*, 1996; Vaarala *et al.*, 1998; Monetini *et al.*, 2002, 2003; Luopajärvi *et al.*, 2008).

Some studies have reported enhanced humoral immune response to various cow's milk proteins in infancy in a subgroup of children fed standard, non-hydrolysed infant formula who later progress to type 1 diabetes (Luopajarvi *et al.*, 2008). Elliott and Martin (1984) reported that addition of skim milk powder to a protein-free diet produced diabetes in the spontaneous diabetes model bio breeding (BB) rats with an incidence of 52%. A semi-synthetic amino acid diet reduced the incidence to 15%. The effect seemed to be established during the early postnatal period (Daneman *et al.*, 1987). However, in another study, diabetogenic food components resulted in diabetes as late as puberty in the BB rat or even later in the non-obese diabetic (NOD) mouse (Coleman *et al.*, 1990). In addition, not only milk ingestion but also introduction of meat and soy protein increased autoimmune diabetes in those animal models (Scott, 1990; Elliott *et al.*, 1988). Other authors have not confirmed the effect of cows' milk protein on diabetes frequency. Malkani *et al.* (1997) reported that neither the addition of BSA to a milk protein-free diet nor the introduction of total milk protein increased the frequency of diabetes in BB Worcester rats. Hydrolysed formula was reported to induce protective effect against type 1 diabetes in NOD mice (Hermitte *et al.*, 1995; Karges *et al.*, 1997a,b). Diabetes could be prevented or reduced in BB rats and in NOD mice by introducing hydrolysed casein (Scott *et al.*, 1985; Elliott *et al.*, 1988; Issa-Chergui *et al.*, 1988; Scott, 1990; Scott *et al.*, 2000) compared to other types of diet. Infants given hydrolysed formula showed a decreased immunological response to cow's milk proteins compared to infants given conventional baby formula (Akerblom *et al.*, 2005).

The ICA69 sequences were shown to cross-react with cows' milk BSA and more particularly with the [152–168] amino acid residue called ABBOS. Elevated serum concentrations of IgG anti-BSA antibodies were detected in diabetic subjects. The majority of these anti-BSA antibodies were specific to the ABBOS peptide and cross-reacted with ICA69 (Beppu *et al.*, 1987; Glerum *et al.*, 1989; Martin *et al.*, 1991; Karjalainen *et al.*, 1992; Pietropaolo *et al.*, 1993; Saukkonen *et al.*, 1994; Levy-Marchal *et al.*, 1995). Significantly increased T cell proliferation responses to BSA and ABBOS were found in children with newly diagnosed

type 1 diabetes (Cheung *et al.*, 1994; Miyazaki *et al.*, 1995). Elevated levels of anti-BSA antibodies were detected in diabetic animals (NOD mice, BB rats) and immunization of NOD mice with BSA or ICA69 generated cross-reactive T cell responses to both Tep69 and ABBOS (Karges *et al.*, 1997). However, the role of BSA in type 1 diabetes was questioned since several authors could not confirm specifically enhanced humoral or cellular responses to BSA or ABBOS in diabetic patients compared to controls (Atkinson *et al.*, 1993; Dosch *et al.*, 1994; Luhder *et al.*, 1994; Ivarsson *et al.*, 1995; Krokowski *et al.*, 1995; Vaarala *et al.*, 1996; Füchtenbusch *et al.*, 1997). In NOD mice fed experimental diets prior to conception, no difference was found in the offspring in diabetes incidence between a standard diet and a milk-free diet (Paxson *et al.*, 1997). Ronningen *et al.* (1998) found no cross-reactivity between BSA and ICA69 in human and animal experiments. Atkinson *et al.* (1993) concluded that anti-BSA antibodies may reflect a general impairment of immunological tolerance. This was associated with a general predisposition to autoimmunity rather than specific β-cell autoimmunity.

It was also hypothesised that exposure to bovine insulin which differs from human insulin only by three amino acids, may break the tolerance to insulin and might cause primary immunisation to insulin in young infants (Vaarala *et al.*, 1998; Paronen *et al.*, 2000; Virtanen *et al.*, 2000; Vaarala 2005; Marttila *et al.*, 2008). Several animal experiments revealed a protective effect of orally administrated human insulin on diabetes type 1 incidence (Atkinson *et al.*, 1990; Zhang *et al.*, 1991; Bergerot *et al.*, 1994; Ploix *et al.*, 1998). After feeding human insulin to 6-week-old NOD mice for one month, Ploix *et al.* (1998) demonstrated a reduction in the severity of insulitis and diabetes incidence. This was due to the development of regulatory T cells suppressing the activity of autoreactive T cell clones. However, in children with HLA-conferred susceptibility to diabetes, administration of nasal human insulin, started soon after detection of autoantibodies, appeared not to prevent or even delay type 1 diabetes (Nantö-Salonen *et al.*, 2008). It has been suggested that addition of human insulin to infant formula in a concentration similar to that present in human milk may be beneficial (Shehadeh *et al.*, 2001). It is speculated whether this addition might protect from the development of type-1 diabetes and lead to even faster gut maturation (Shehadeh *et al.*, 2001).

The possibility that molecular mimicry occurs between casein and islet antigens (p69, carboxypeptidase, GLUT-2) has also been proposed. Cell-mediated immune responses to β-casein were observed in patients with diabetes of recent onset compared to healthy controls. It was postulated that a sequence homology between residues 63–67 of bovine β-casein and 415–419 of the β-cell-specific glucose transporter GLUT-2 could be the pathogenic mechanism (Inman *et al.*, 1993; Cavallo *et al.*, 1996a,b). Elevated levels of antibodies to β-casein were demonstrated in some patients with type 1 diabetes but not in others (Wasmuth *et al.*, 1995; Elliott *et al.*, 1996a; Monetini *et al.*, 2002, 2003). Ellis *et al.* (1998) found cellular immune responses to β-casein in type 1 diabetes compared to autoantibody negative healthy control subjects. T cell immune response to β-casein was the same in the diabetic patients and their autoantibody negative relatives. The authors concluded that individuals genetically prone to autoimmunity may be deficient in developing tolerance to dietary antigens. The role of β-casein as a causative factor in diabetes development remains unclear. Animal experiments have produced contrasting results concerning the diabetogenic potential of casein. Some

studies demonstrated a protective effect of whole casein as a substitute for skim milk (Scott *et al.*, 1985; Issa-Chergui *et al.*, 1988; Scott, 1990), whereas others reported that introduction of β-casein as the only source of protein before weaning leads to the development of diabetes in NOD mice (Elliott *et al.*, 1988).

Beta-lactoglobulin (β-lg) is the major whey protein in cow's milk (Rudloff and Kunz, 1997) but is not expressed by the human mammary gland. It was demonstrated that this protein is involved in allergic reactions to cow's milk (Sabikhi, 2007). Enhanced humoral and cellular immune response to β-lg was also demonstrated in some diabetic patients compared with control subjects (Dahlquist *et al.*, 1992; Savilahti *et al.*, 1993; Saukkonen *et al.*, 1995; Vaarala *et al.*, 1996; Vähäsalo *et al.*, 1996). A recent study points out a possible cross-reaction between anti-β-lg and the human protein glycodelin (PP14), a T cell modulator, undermining T cell regulation of beta cells in infancy (Goldfarb, 2008). Cellular responsiveness to β-lg was not associated with HLA-DQB1 risk alleles, implying that immune response to the protein does not reflect the accumulation of these HLA alleles in the patients with type 1 diabetes.

The link between milk proteins and type 1 diabetes remains unclear. When genetic risk determinants were included, antibodies to BSA, β-lg, whole cow's milk and islet cell antibodies were not independently associated with the risk of type 1 diabetes in a multivariate, logistic regression analysis (Saukkonen *et al.*, 1995; Vähäsalo *et al.*, 1996; Saukkonen *et al.*, 1998; Redondo *et al.*, 1999).

4.5.4.4 β-casein variants, BCM7 and type 1 diabetes

After *in vitro* digestion with intestinal enzymes, BCM7 has been shown to be released at a higher level from β-casein variants A^1 and B than from the A^2 variant (see Chapter 3.2). A hypothesis has been proposed that the opioid peptide BCM7 might contribute to the impairment of the development of gut-associated immune tolerance in IDDM genetically predisposed individuals. It might thus act as an adjuvant in the autoimmune reaction involved in the destruction of β-cells in prediabetic subjects (Elliott *et al.*, 1997; Hartwig *et al.*, 1997; Åkerblom and Knip 1998; Elliott *et al.* 1999). One study has shown that antibodies against casein variants occur both in normal controls and in patients with IDDM. However, patients with IDDM and their siblings showed increased amounts of anti-casein A^1 antibodies in serum samples, whereas their parents and control persons showed increased amounts of anti-casein A^2 antibodies in serum samples (Padberg *et al.*, 1999). Although BCMIR can be present in the intestinal lumen, the specific release of BCM7 from β-casein variants A^1 and B has not been demonstrated *in vivo* in humans (Svedberg *et al.*, 1985) (see chapter 3). The diabetogenicity of β-casein A^1, A^2 and B variants has been evaluated in animal studies and in ecological studies in humans.

Two animal studies have investigated the differences in the diabetogenic effect between A^1 and A^2 casein (Elliott *et al.*, 1997; Beales *et al.*, 2002). Both feeding studies combined casein with a soy preparation in NOD mice. The first study which was not published in a peer-reviewed journal, found a higher incidence of diabetes after feeding NOD mice with A^1 β-casein compared to A^2 β-casein (Elliott *et al.*, 1997). In contrast, the second study was published in an international, peer-reviewed journal and did not confirm these results (Beales *et al.*, 2002). In this study, the authors showed that the combination of A^2 with soy protein

(Prosobee (PS), soy based formula) resulted in a lower incidence of diabetes in the BB rat, whereas the combination of A^1 and A^2 with Pregestimil (a hydrolysed milk formula) did not result in a difference. However, the authors concluded that A^1 β-casein was more diabetogenic than A^2 β-casein in PS-fed BB rats, and that although milk caseins are unlikely to be exclusive promoters of type 1 diabetes, they could enhance the induction of diabetes in some cases. It should be taken into account that soy proteins have been suggested to contribute to the development of diabetes, as well as Pregestimil. In the study of Beales *et al.* (2002), wheat was shown to be much more diabetogenic than other protein combinations. The studies performed to date do not allow a conclusion on a direct effect, if any, of β-casein A^1 or A^2 on the development of diabetes in animal models.

The first group of human ecological studies tested the correlation between the estimated per capita consumption of β-casein A^1 and A^2 and the incidence of type 1 diabetes. A study on type 1 diabetes incidence in 0 to 14-year-old children from 10 countries or areas, including all the Nordic countries, compared the national cow's milk protein consumption along with milk protein polymorphism. They observed that total protein consumption did not correlate with diabetes incidence but consumption of β-casein A^1 alone or in combination with β-casein B did correlate with IDDM incidence (Elliott *et al.*, 1999). The calculated consumption of β-casein A^1 across 16 countries was reported to correlate strongly with the incidence of type 1 diabetes in males under 15 years (McLachlan, 2001). A larger ecological study, including 19 countries from all over the world, reported similar associations (Laugesen and Elliott, 2003). However, the difference in the incidence of type 1 diabetes can also be explained by other not reported confounding factors as already outlined for CVD, since the authors used the same estimated β-casein variants intakes for their statistical analyses (in Chapter 4.5.3.3). Moreover, as discussed in Chapter 2.3, the relevance of data on β-casein allele distribution and the actual level of specific protein variants in milk and dairy products appear difficult to interpret.

A further ecological study evaluated the correlation between β-casein A^1 and B intake and type 1 diabetes incidence in the group of Nordic countries: Iceland, Denmark, Norway, Sweden and Finland (Thorsdottir and Reykdal, 1997; Birgisdottir *et al.*, 2002, 2006). The Icelandic population has a genetic origin similar to that of the other Nordic countries, except Finland (Wijsman, 1984). No major differences in HLA allele distribution between Icelandic and Norwegian diabetic patients or control groups could be found (Backman *et al.*, 1998), although the incidence of type 1 diabetes in Norway has been reported to be about twice as high as in Iceland (Joner and Sovik 1989; Helgason *et al.*, 1992). No association was found between cow's milk consumption in infancy and the development of type 1 diabetes in the Icelandic population (Thorsdottir *et al.*, 2000) whereas many studies have suggested that early ingestion of cow's milk products in infancy is associated with an increased risk for the development of type 1 diabetes (Borch-Johnsen *et al.*, 1984; Mayer *et al.*, 1988; Virtanen *et al.*, 1991; Kostraba *et al.*, 1992, 1993; Gerstein 1994; Patterson *et al.*, 1994). The study of Birgisdottir *et al.* (2006), focusing on children, reported a lower average consumption of β-casein A^1 and B variants among 2-year old children in Iceland (approximately 1.7 g/day) compared to the same age group in the Scandinavian countries (Denmark, Norway, Sweden, Finland) (approximately 2.7 g/day) where the incidence of type 1 diabetes is higher. The

study also reported a positive correlation between the estimated intake of β-casein A^1 among 2 year olds in the different Nordic countries and the incidence of type 1 diabetes.

However, a comprehensive evaluation of possible confounders and uncertainties is needed in order to assess the strenght of the above correlations. Dietary intakes in infancy and childhood may be expected to have a large inter-individual and age-related variation. Given the general limitations of ecological studies, the possibility that the children with type 1 diabetes had a low intake of β-casein A^1 when compared to other children in the same country, cannot be rejected. Therefore, the possibility that other confounding factors are responsible for the disease, cannot be rejected by an ecological study. In the first step Birgisdottir *et al.* (2006) restricted the observed population to more homogeneous sex and age groups, in particular children at 2 years of age, and male as well as female adolescents aged from 11 to 14 years. The obtained correlation was only significant (to α=5%) for the youngest children and β-casein A^1. Some methodological problems can be highlighted, however. While the best available comparable data were used (Birgisdottir *et al.*, 2006), the incidence rates of type 1 diabetes covered the period up to the middle of the 90's. However the consumption data for the different countries were taken between 1980 and 1999, while information regarding the β-casein A^1 and B were from early and late 1990s.

In addition, the exposure to β-casein A^1 versus A^2 of IDDM and non-IDDM subjects needs to be considered. The β-casein A^1+B content in milk produced in Iceland and in other Nordic countries in the late 90s was estimated to be 38.4 % and 48.7%, respectively (Thorsdottir *et al.*, 2000). As a consequence, it can be seen that the IDDM and non-IDDM groups were both exposed to significant levels of β-casein A^1+B. As a consequence, it seems difficult from an immunological viewpoint to explain the difference in incidence of IDDM by such a small difference in the estimated exposure to β-casein A^1+ B.

In summary, no specific putative factor could be identified in the reviewed literature for which a cause-effect relationship with IDDM could be proven.

CONCLUSIONS

Conclusions on bioactive peptides and source proteins

- Proteins, including those present in the diet, are a potential source of a wide range of biologically active peptides, including some with affinity for opioid receptors.
- The release of BCM7 through enzymatic digestion of bovine β-casein is dictated by the primary amino acid sequence, which is dependent on the genetic variant of this protein.
- The allele frequency distribution of β-casein is determined by bovine breed and population.
- Changing selection targets in the last decades has resulted in changes in bovine breed composition in most European countries. While no detailed information is available, it is likely that these changes in breed composition have had an impact on milk composition, including different milk protein variant concentration and type.

- Taking into account the uncertainty in milk composition and the diverse geographical origin of dairy products and ingredients across Europe, insufficient information is currently available on the exposure of individual consumers to different β-casein variants.

Conclusions on opioid peptides in food and their formation during digestion

- The presence of BCMs or related peptides in unprocessed milk obtained from healthy cows has not been conclusively demonstrated.
- There is a substantial body of evidence indicating that different proteolytic systems involved in fermented milk or cheese manufacture can potentially hydrolyze β-casein to BCM7 or other BCMs and further degrade these peptides to shorter-chain peptides and even amino acids.
- Little or no information is currently available about the actual BCM levels which different proteolytic systems may generate in fermented or enzyme treated milk products. Furthermore, the stability of these peptides, once generated, is variable.
- Technological conditions such as heat treatments applied in industrial dairy processing do not seem to influence the occurrence of BCMs in the final products or influence their formation during subsequent digestion.
- The role of proteolytic systems in favoring the release of BCMs during SGID or *in vivo* digestion, eventually through the formation of BCM precursors, has not been clarified. However, there are indications that a successive action of several digestive enzymes is involved.
- Evidence about the occurrence of BCMs in commercial infant formulas is currently inconclusive. However, the subsequent formation of certain BCMs in infant formulae after SGID (with multiple enzyme activities) has been demonstrated.
- No current studies report quantitative values for BCMs formed during the *in vivo* digestion of dairy products.

Conclusions on molecular interactions, general biological effects and fate of food-derived peptides

- Animal data clearly support the idea that β-casomorphins, including BCM7, can act as opioid receptor agonists, probably acting via μ-type opioid receptors.
- At a molecular level, in comparison to medicinal and endogenous opioids, bovine BCM7 does not seem to be a very potent opioid ligand.
- Opioid effects were only observed *in vivo* in animal studies following intra-peritoneal (i.p.) or intra-cerebro-ventricular (i.c.v.) administration.
- Relatively little is known on the mechanisms of transfer of intact peptides longer than 3 amino acids across the intestinal barrier; however, if this transport occurs, then the extent is very low and passive diffusion is the most likely transfer mechanism.

- β-casomorphin immunoreactive material in blood has been reported in two studies within neonatal dogs and calves. However, the presence of β-casomorphin molecules in blood after intake of milk or casein has not been established in *in vivo* studies.
- Opioid peptides, including β-casomorphin 4, -5 and -7 are highly sensitive to hydrolysis by dipeptidyl peptidase IV thereby strongly limiting or preventing the transfer of these peptides in an intact form across the intestinal mucosa and the blood-brain barrier.
- Available data suggest that in principle, transport of food-derived peptides and proteins across the human intestinal mucosa is possible. However, quantitative data on this phenomenon are lacking. In certain cases such as in neonates and adults with specific diseases, intestinal permeability has been reported to be significantly increased.

Conclusions on organ and system specific actions

- Food-derived peptides, including casomorphins, can have different effects in the intestinal lumen and the intestinal mucosa, such as regulatory effects on gastro-intestinal motility and on gastric and pancreatic secretions.
- Many studies report effects of β-casomorphins on the CNS following i.p. or i.c.v. administration in animals.
- A possible link between BCM intake and sudden infant death syndrome (SIDS) has been suggested in some publications. However, no clear evidence for such a relationship was found during the review.
- A link between casein-derived peptides and autism in subjects with increased intestinal permeability has been suggested. However, recent data do not provide any support for such a relationship.
- It has been suggested that BCM7 might be atherogenic through an oxidative action on LDL. This mechanism was originally proposed in a single report, however, it has not been confirmed by later studies.
- The possibility that BCM7 could contribute to an increased risk for atherosclerosis has also been suggested from a study in a rabbit model. This study concluded that β-casein A^1 would be atherogenic, in contrast to β-casein A^2. However, the validity of the experimental model and the extrapolation to atherosclerosis in human were not regarded as convincing.
- Some ecological studies have linked the intake of BCM7 with cardiovascular disease mortality. However, these ecological studies did not account for several confounding factors. In addition, recent large cohort studies led to opposite conclusions.
- Two human intervention studies comparing diets containing β-casein A^1 and A^2 did not show a correlation between the estimated β-casein A^1 consumption and development of certain biomarkers for cardiovascular disease. A limitation of these studies was the small number of subjects and the short intervention period.
- Overall, this review process did not find any strong evidence for a link between the consumption of β-casein A^1 and an increased risk for CVD in humans.

- Insulin dependent diabetes mellitus (IDDM) is recognised as a multifactorial autoimmune disease; however, its pathogenesis is unclear.
- Autoantibodies have been found in IDDM patients, but the role of autoantibodies in type 1 diabetes is currently not known. These autoantibodies have not been proven to be directly involved in either tissue destruction or disease progression.
- The development of IDDM is the result of a combination of genetic predisposition and environmental risk factors where genetic predisposition is a necessary condition.
- Many dietary risk factors of IDDM have been indicated, including short duration of breast feeding, gluten, soy, cow's milk and solid foods at young age.
- Several different milk proteins or peptides derived from these proteins have been proposed as possible diabetogenic factors. The mechanism most often suggested is immunological.
- The diabetogenicity of β-casein A^1, A^2 and B has been evaluated in animal studies and ecological studies on humans. Animal studies have presented contradictory results.
- Some ecological studies have linked the intake of BCM7 with IDDM. Ecological studies have the shortcoming of being unable to establish cause-effect relationship and they cannot adjust for possible confounding factors. They are at best indicating a hypothesis but cannot provide a proper base to demonstrate a cause-effect relationship.
- The correlations suggested by these studies may become very weak when taking into account the uncertainties in individual consumption, in the β-casein variant composition, and in some countries also the IDDM incidence rate.
- The difference in content of β-casein A^1+B in milk produced in countries with high or low prevalence of IDDM appears relatively small and does not explain, from an immunological point of view, the difference in incidence of IDDM across countries.

RECOMMENDATIONS

Based on the present review of available scientific literature, a cause-effect relationship between the oral intake of BCM7 or related peptides and aetiology or course of any suggested non-communicable diseases cannot be established. Consequently, a formal EFSA risk assessment of food-derived peptides is not recommended.

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APPENDIX
DISEASE RELATED STUDIES

	Risk factor	Risk	Study type	No of ref.
A	Casein intake	Heart diseases	Ecological	2
B	Casein intake	Diabetes type 1 / IDDM	Ecological	5
C	Nutrition	Diabetes type 1 / IDDM	Ecological	2
D	Infant diet	Diabetes type 1	Case Control	8
E	Dairy intake	Diabetes type 2	Cohort	1
F	Immune response against Casein	Diabetes type 1 / IDDM	Case Control	10
G	BCM7	Skin response	Trial	1
H	Infant diet	Antibodies against casein	Trial, Cohort	3
I	Casein intake	Measures of cardiovascular disease	Trial	1
J	Milk consumption	Cardiovascular disease	Cohort	5
K	Casein intake / Antibodies against casein	Autistic syndrome	Various	7
L	Protein uptake / Antibodies against casein	Multiple sclerosis	Case Control	1
M	Dairy consumption	Parkinson's disease	Cohort	3

A – casein intake and heart disease

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B – casein intake and IDDM

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C – nutrition and IDDM

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D – infant diet and IDDM

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E – dairy intake and diabetes type 2

Choi, H. K., W. C. Willett, et al. (2005). "Dairy consumption and risk of type 2 diabetes mellitus in men - A prospective study." Archives of Internal Medicine 165(9): 997-1003.

F – immune response to casein and IDDM

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G – BCM7 and skin response

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H – infant diet and antibodies against casein

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I – casein intake and CVD markers

Chin-Dusting, J., J. Shennan, et al. (2006). "Effect of dietary supplementation with beta-casein A1 or A2 on markers of disease development in individuals at high risk of cardiovascular disease." British Journal Of Nutrition 95(1): 136-144.

J – milk consumption and CVD

Elwood, P. C. (2005). "Milk and cardiovascular disease: a review of the epidemiological evidence." Australian Journal of Dairy Technology 60(1): 58-60.

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Ness, A. R., G. D. Smith, et al. (2001). "Milk, coronary heart disease and mortality." Journal Of Epidemiology And Community Health 55(6): 379-382.

K – casein intake/antibodies against casein and autism

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Knivsberg, A. M. and K. L. Reichelt (2004). "Autistic syndromes and diet: A single blind study." Focus on Autism Research: 213-245.

L – protein intake/related antibodies and multiple sclerosis

Reichelt, K. L. and D. Jensen (2004). "IgA antibodies against gliadin and gluten in multiple sclerosis." Acta Neurologica Scandinavica 110(4): 239-241.

M – dairy intake and Parkinson's syndrome

Chen, H., E. O'Reilly, et al. (2007). "Consumption of dairy products and risk of Parkinson's disease." Am J Epidemiol 165(9): 998-1006.

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GLOSSARY/ ABBREVIATIONS

BCM7 (BCMn)	β-casomorphin-7 (β-casomorphin-n)
BCMIR	β-casomorphin immunoreactive material
BCMs	β-casomorphins
BSA	Bovine serum albumin
CEP	Cell envelope proteinases
CN	Casein
ConA	Concavalin A
CVD	Cardiovascular disease
ESI	Electro spray ionisation
GI	Gastrointestinal
HDL	High density lipoprotein
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
i.c.v.	Intra-cerebro-ventricular injection
i.p.	Intra-peritoneal injection
ICA	Islet Cell Autoantigen
IDDM	Insulin-dependent diabetes mellitus
IUPHAR	International Union of Basic and Clinical Pharmacology
LAB	Lactic acid bacteria
LAP	Leucine amino peptidase
LC	Liquid chromatography
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MALDI	Matrix assisted laser desorption ionisation
MS	Mass spectrometry (also as detection/identification tool connected with LC)
PepX	X-prolyldipeptidyl aminopeptidase
PHA	Phytohemagglutinin
SCC	Somatic cell count
SGID	Simulated gastro intestinal digestion
TOF	Time of flight
UF	Ultra filtration
UHT	Ultra high temperature / short time heat treatment
UV	Ultra violet