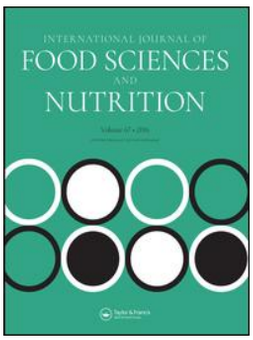

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RESEARCH ARTICLE

Unravelled facets of milk derived opioid peptides: a focus on gut physiology, fractures and obesity

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ABSTRACT

Beyond being a source of key nutrients, bovine milk influences physiological functions by synthesising bioactive peptides during the process of digestion. Some of the claimed negative health outcomes associated with milk consumption, such as cardiovascular diseases and type 1 diabetes may be attributed to an opioid peptide, beta-casomorphin-7 (BCM-7), derived from A1 beta-casein. BCM-7 exerts its function by binding to the μ-opioid receptors in the body. It is hypothesised that activation of the μ-opioid receptors in the gut can alter gut microbial composition, impair gut barrier integrity and bile acid metabolism, in addition to increasing gastrointestinal transit time and gut inflammation. Further, it is hypothesised that BCM-7 may influence fractures and obesity via μ-opioid receptor pathways. In conclusion, it appears that BCM-7 might have multiple functions pertinent to human health; however, the evidence is limited and warrants further pre-clinical and clinical studies for hypothesis confirmation.

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Milk; beta-casomorphin-7; gut microbiota; gut inflammation; fractures; obesity

Introduction

Bioactive peptides are short-chain peptides derived from the intact protein and have multiple physiological functions (Meisel 1998; Nguyen et al. 2015). Milk and dairy products have been identified as one of the largest contributors of bioactive peptides among food-derived peptides (Moller et al. 2008). Bioactive peptides derived from dairy protein possess several important physiological activities including immunomodulatory (Gill et al. 2000), anti-hypersensitive (Takano 2002), antimicrobial (Meisel 1998), osteoprotective (Tsuchita et al. 1993) and opioid functions (Brantl et al. 1979; Stefanucci et al. 2018).

The opioid peptides derived from milk protein have placed milk consumption under scrutiny due to the evidence suggesting that these peptides may be linked to the pathophysiology of some diseases (Sun et al. 1999; McLachlan 2001; Laugesen and Elliott 2003). Lactose intolerance and milk protein allergies are the most recognised concerns associated with consuming milk. However, apart from these, there are epidemiological data suggesting that consuming milk containing a particular protein variant, A1 beta-casein, may predispose to a range of diseases such as cardiovascular diseases (Laugesen and Elliott 2003), type 1 diabetes (Elliott et al. 1999) and neurological disorders (e.g. autism, schizophrenia and sudden infant death syndrome) (Sun et al. 1999, 2003).

Casein is the predominant form of milk protein and contains four major subsets. Of the four variants, beta-casein forms the basis for the A1/A2 hypothesis. A single point mutation in the gene coding for beta-casein occurred 10,000 years ago in Holstein European herds and caused a change in the amino acid from proline to histidine and produced the A1 beta-casein variant of A2 beta-casein (Kamiński et al.

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2007; Haq, Kapila, Shandilya, et al. 2014). Enzymes in gut digest A1 and A2 beta-casein differently because of the structural dissimilarity of those beta-caseins. The A1 beta-casein fraction in milk produces beta-casomorphin-7 (BCM-7) while A2 beta-casein produces BCM-9 (Jinsmaa and Yoshikawa 1999; Haq, Kapila, Shandilya, et al. 2014). BCM-5 can be produced by further catabolism of BCM-7; these peptide fragments are characterised as beta-casomorphins (BCMs) (Pal et al. 2015). Cieślińska et al. (2012) showed that BCM-7 is synthesised in only minor quantities from milk exclusively containing A2 beta-casein variant compared to the milk containing both beta-casein variants. Other dairy products such as cheese and yogurt are also reported as sources of BCMs that parallel their A1/A2 milk lineages (De Noni and Cattaneo 2010).

The potential negative physiological implications of BCM-7 have been extensively discussed in literature in the areas of ischaemic heart disease (Laugesen and Elliott 2003), type 1 diabetes (Elliott et al. 1999), psychomotor development (Kost et al. 2009) and sudden infant death syndrome (Sun et al. 1999, 2003). On the other hand, some studies have shown putatively beneficial aspects of BCM-7 in e.g. mucous secretion (Trompette et al. 2003) and cellular immunity (Elitsur and Luk 1991). However, the literature lacks information on how BCMs may alter certain gut parameters (e.g. gut microbiota, gut epithelial integrity and bile acid metabolism), fractures (bone metabolism) and obesity. Herein, the purpose of this review is to analyse the overlooked potential functions of BCMs and to hypothesise potential mechanistic pathways by which they might play a role in the pathogenesis of disease.

Methods

Literature addressing topics such as (1) A1/A2 beta-casein, (2) μ-opioid receptors, (3) milk consumption and gut physiology, fractures and obesity and (4) implication of morphine on gut, fractures and obesity were searched in Medline and Google Scholar. Peer-reviewed original research work, reviews and theses written in English were included, whereas conference abstracts and grey literature were excluded. The searched articles were further analysed for the suitability to be included in this manuscript based on the manuscript focus. Articles on both animal and human studies according to the following criteria were included: (1) Studies assessing the effects of A1/A2 beta-caseins on gut physiology; inflammation, fractures and obesity; (2) Observational studies assessing the impacts of milk consumption on gut physiology, fractures and obesity; and (3) Studies assessing the effects of morphine on gut physiology, fractures and obesity.

Interaction of beta-casomorphins with the body

BCMs are μ-opioid receptor ligands and are classified as "atypical opioid peptides". Brantl et al. (1981) reported that BCM-4/5/6/7 demonstrated opioid activity in an opiate receptor binding assay and these peptides produced naloxone-reversible analgesia. Naloxone is a morphine antagonist and thus reverses the action of morphine. BCM-5/7 are the potent forms of BCMs and show higher degree of affinity towards μ-opioid receptors, whereas BCM-9 has the least affinity (Jinsmaa and Yoshikawa 1999; Haq, Kapila, Shandilya, et al. 2014). Opioid receptors belong to the superfamily of G-protein coupled receptors, which are categorised as mu (μ), delta (γ) and kappa (κ) receptors (Chan 2008). For the most part, opioid receptors are found in the central nervous system (CNS) (Chan 2008). However, opioid receptors can also be found in the peripheral nervous system, the gastrointestinal (GI) tract, the myenteric plexus of the enteric nervous system and the immune system (T cells, B cells and macrophages) (Holzer 2009; Ninkovic and Roy 2013). Furthermore, it is reported that opioid receptors are found in bone cells (Rosen et al. 1998). Opioid receptors are thus distributed across many parts of the body allowing opioids to exert their functional effects from pain management to bone metabolism, thus indicating the pluripotent physiological effects of opioids in human health (Figure 1).

In order to demonstrate opioid activity, BCMs should be yielded in pharmacological concentrations that are sufficient to elicit physiological response (Boutrou et al. 2013; Pal et al. 2015) and be able to bind to the opioid receptors. Boutrou et al. (2013) showed that 4 mg of BCM-7 was released in the human jejunum, 2 h after digestion of 30 g of casein, which is consistent with pharmacological effects. On the other hand, demonstration of the crossing of the gut epithelia into circulation is essential in order to explain the functional effects of the BCMs. The potential of BCMs to transfer across intestinal epithelia has been demonstrated in *in vitro* experiments (Iwan et al. 2008; Sienkiewicz-Szlapka et al. 2009), in animals (Singh et al. 1989) and in infants (Wasilewska et al.

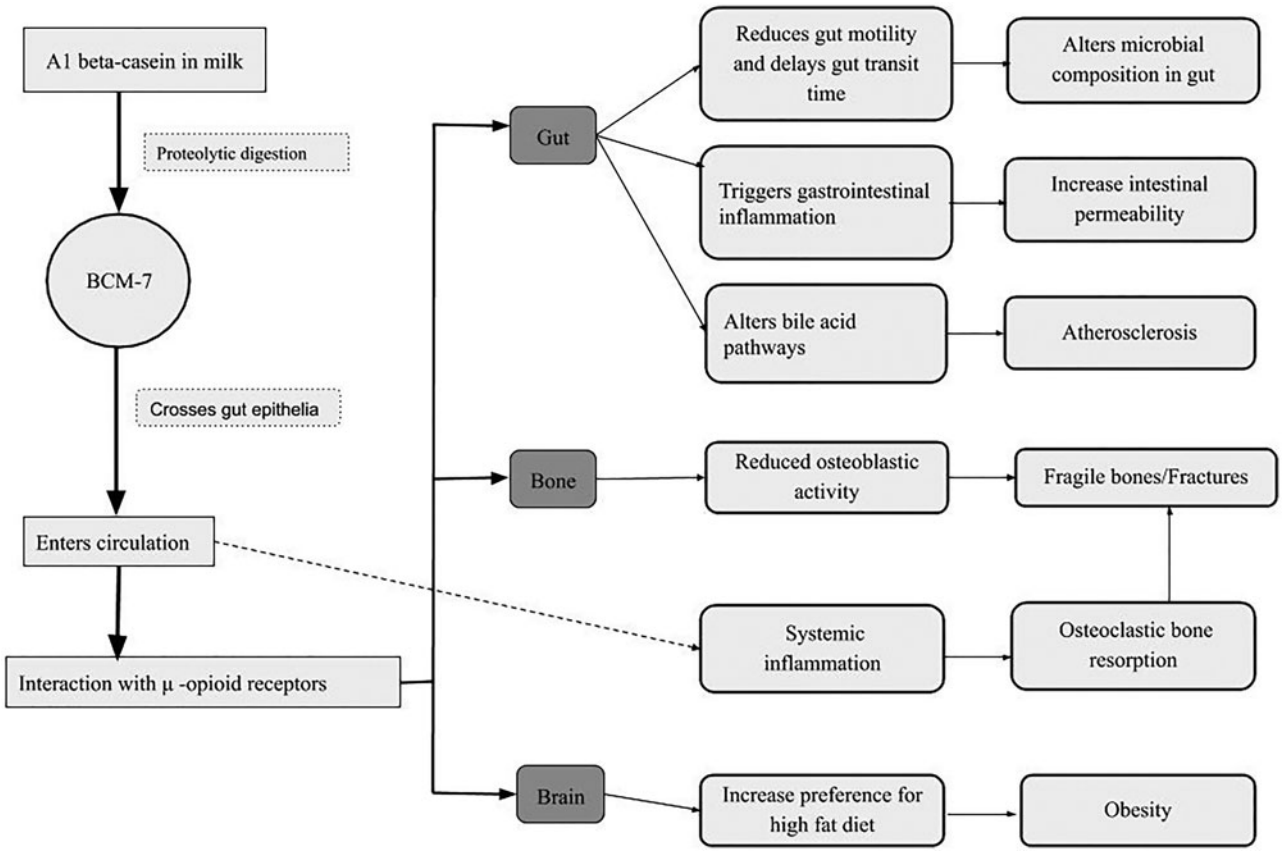

Figure 1. Potential pathways by which milk opioid peptides may influence physiological functions.

2011); however, evidence in adults is lacking. It is postulated that BCMs could be transferred via paracellular pathways across intestinal epithelia – i.e. the intercellular space between cells (De Noni et al. 2009) – and the process may occur swiftly in infants due to the immature gut lining. However, according to the European Food Safety Authority (EFSA) report published in 2009, existing *in vivo* evidence was not sufficient to confirm the transfer of BCM-7 across intestinal epithelia and the topic warranted more investigations (De Noni et al. 2009). Nevertheless, recently published studies have shown that casein-derived peptides can be transferred across the intestinal epithelia by measuring postprandial plasma BCM-7 levels (Deth et al. 2016; Janssen Duijghuijsen et al. 2016; Jianqin et al. 2016).

The potential impacts of milk opioid peptides on gut physiology

The presence of opioid receptors in the GI tract may be responsible for the bowel dysfunction associated with opioid administration (Kurz and Sessler 2003). Opioids alter GI functions through opioid signalling pathways. The gut contains highly-specified binding sites for opioid drugs and opioid peptides. Mostly μ-opioid receptors are situated in enteric neurons (Claustre et al. 2002) and intestinal epithelial cells (Zoghbi et al. 2006). In the following section, we will discuss possible implications related to the activation of μ-opioid signalling pathways by the A1 beta-casein on GI transit time, gut microbial composition, gut inflammation, gut epithelial integrity and bile acid metabolism (Table 1).

Gastrointestinal transit time and gut microbial composition

A number of animal studies have demonstrated the effect of caseins or their derivatives in delaying GI motility via μ-opioid receptor pathways (Becker et al. 1990; Defilippi et al. 1995; Barnett et al. 2014). An experiment using rat pups explored the potential for bovine casein to alter GI motility and showed that BCMs produced during the digestive process caused a delay in GI transit (Daniel et al. 1990). This effect was reversed by naloxone, thus providing evidence for the direct interaction of casomorphins with gut opioid receptors (Daniel et al. 1990). Another study in dogs showed the *in vivo* effect of casein administration on

Table 1. Implications of milk beta-caseins variants and their derivatives in animal and human models.

	Animal studies			
	Animal model	Study product/compound	Outcome of interest	Results
Defilippi et al. (1995)	Canine	Casein diet and soy diet	Motor activity of small bowel	The amplitude and frequency of small bowel reduced in casein diet
Becker et al. (1990)	Mice	Subcutaneous administration of morphine, beta-casomorphin derivatives	GI transit time	GI transit time reduced with morphine and beta-casomorphin derivatives
Barnett et al. (2014)	Rat	A1 diet with A1 skim milk powder and A2 diet with A2 skim milk powder	GI transit time MPO activity DPP-4 activity	A1 diet increased the GI transit time, MPO and DPP activity
Daniel et al. (1990)	Rat	Whey protein suspension Casein suspension BCM-4 BCM-5	GER GI transit time	Longer GER and GI transit time were observed with casein suspension, BCM-5
Haq, Kapila, Sharma, et al. (2014)	Albino mice	A1/A1 extract A1/A2 extract A2/A2 extract	MPO activity MCP-1, IL-4 IgE, IgG, sIgA, IgG1 and IgG2a TLR-2, TLR-4	Increased MPO activity, inflammatory cytokines, antibodies, and toll-like receptors in A1/A1 and A1/A2 diet fed rats
Haq, Kapila, and Saliganti (2014)	Mice	BCM-5 BCM-7	MPO activity Inflammatory cytokines (MCP-1, IL-4) Antibodies (IgE, IgG, sIgA, IgG1 and IgG2a) Toll-like receptors (TLR-2, TLR-4)	Increased MPO activity, inflammatory cytokines, antibodies, and toll-like receptors in A1/A1 and A1/A2 diet fed rats gavage BCM-5/7
	Human studies			
	Study design	Study products	Outcome measures	Results
Ho et al. (2014)	An 8-week double blinded randomised cross-over study with 41 healthy males and females	A1 milk and A2 milk (750 ml/d)	Stool consistency GI symptoms	Higher stool consistency and abdominal pain in the group which consumed A1 milk
Jianqin et al. (2016)	An 8-week double blinded randomised cross-over study with 45 males and females with self-reported intolerance to cow's milk	A1 milk and A2 milk (500 ml/d)	Post dairy digestive discomfort GI transit time Faecal SCFA Blood markers of inflammation (CRP, IL-4, IgG, IgE)	Increased post dairy digestive discomfort, GI transit time, reduced SCFA in faeces and augmented serum markers of inflammation were reported in the group which consumed A1 milk
He et al. (2017)	A double-blinded randomised cross-over study with a total of 600 males and females with cow's milk intolerance	A1 milk and A2 milk (300 ml/d)	GI symptoms (borborygmus, flatulence, bloating, abdominal pain, stool frequency, and stool consistency)	Higher symptoms score for GI symptoms was observed in the group which consumed A1 milk

GI: gastrointestinal; GER: gastric emptying rate; MO: myeloperoxidase; MCP-1: monocyte chemotactic protein-1; IL-4: interleukin- 4; IgE, IgG, sIgA, IgG1 and IgG2a: antibodies; TLR-2/4: toll-like receptor-2/4; DPP-4: jejunal dipeptidyl peptidase-4; A1 milk: contains A1 & A2 beta-casein; A2 milk: contains only A2 beta-caseins; SCFA: short chain fatty acids; CRP: C-reactive protein.

small intestinal measures, such as force and contraction frequency and concluded that casein significantly reduced these parameters and, again, that this effect was abolished by naloxone (Defilippi et al. 1995). Similarly, another experiment studied the effect of A1 versus A2 beta-casein on GI transit time in Wistar rats and reported that GI transit time was delayed in A1 beta-casein fed rats compared to A2 beta-casein fed rats (Barnett et al. 2014). Administration of naloxone neutralised the effects of the A1 diet on transit time, but no effects were seen in rats fed with the A2 diet, indicating that A1, but not A2, influences GI function by reducing the transit time via the opiate system (Barnett et al. 2014). However, this evidence is based on animal studies and may not be generalisable to humans.

There are only few human studies assessing the impacts of the types of beta-casein variants on GI transit time either directly or indirectly (Ho et al. 2014; Jianqin et al. 2016). The randomised 2*2 cross-over trial led by Jianqin et al. (2016) in 45 Chinese subjects with perceived lactose intolerance showed that the group consumed milk containing both A1 and A2 beta-casein variants had significantly longer colonic and whole gut transit time (measured by a smart pill) compared to the group consuming milk containing only A2 beta-casein. However, it is worth noting that the smart pill was not used at the baseline of the study and this limitation precluded the authors from determining whether the milk containing only the A2 beta-casein type influenced GI transit time. Another randomised controlled trial in 41 healthy Australian subjects assessed the effects of beta-casein variants on several GI symptoms, however, they did not directly measure GI transit time (Ho et al. 2014). The trial reported significantly higher stool consistencies (measured by Bristol Stool Scale) in the group that consumed milk containing both A1 and A2 beta-caseins (Ho et al. 2014). As higher stool consistencies are related to increased GI transit time (Lewis and Heaton 1997; Jaruvongvanich et al. 2017), this is concordant with the idea that the type of beta-casein variants might have affected GI transit time.

Additionally, there are other studies to show that increased milk consumption is associated with constipation regardless of the type of beta-caseins. Andiran et al. (2003) found associations between milk consumption, constipation and anal fissures. In this observational study, they revealed a higher incidence of chronic constipation and anal fissures in children (aged 4 months–3 years) who consumed higher amount of cow's milk and were breast-fed for a shorter period of time, than children who were symptom-free. Further, an open-label cross-over study that compared cow's milk and rice milk consumption in 69 children showed a positive association between cow's milk consumption and constipation (Irastorza et al. 2010). More than one third of children who participated in this study developed constipation following cow's milk ingestion, which resolved within 1–5 d after withdrawal of cow's milk. The authors of this study suggested a diet free of cow's milk as a therapeutic option for chronic constipation in childhood (Irastorza et al. 2010). However, the study design did not allow for blinding of participants or investigators, making the study outcome harder to interpret. Taken together, these findings suggest that higher intake of bovine milk may be associated with constipation, although mechanisms underlying the association were not elucidated. However, in theory, the evidence from animal studies and human trials suggests that the association may be dependent on the type of milk protein and the opioid peptides they produce.

Interplay between gastrointestinal transit time and gut microbial composition

The human gut is colonised by 10–100 trillion microbes, each of which has preferred nutritional substrates that – if abundant or deficient – drive population preponderances (Ursell et al. 2012; Boulange et al. 2016). The majority of bacteria are located in the large intestine. Gut bacteria digest complex carbohydrates and produce important bioactive factors such as short-chain fatty acids (SCFAs), vitamins and neurotransmitters and control the growth of harmful bacteria (Bik et al. 2018). The gut microbiome is initially colonised at birth and in early life (Kundu et al. 2017) and its composition during life is influenced by many endogenous and exogenous factors (Linares et al. 2016). Transit time has been shown to influence the microbial composition and fermentation patterns in the colon (Kashyap et al. 2013). Some studies show that prolonged transit time creates an imbalance in gut microbial composition, or dysbiosis (Stephen et al. 1987; Nieuwenhuijs et al. 1998), by facilitating bacterial overgrowth, especially in the small intestine, which may also lead to bacterial translocation into circulation (Nieuwenhuijs et al. 1998; Balzan et al. 2007).

The involvement of μ-opioid receptor signalling pathways in gut microbial dysbiosis has been studied in animal models. A rat study demonstrated that prolonged transit time induced by opioids increased bacterial overgrowth and consequently disseminated intestinal luminal bacteria to the peripheral tissues

(Runkel et al. 1993). Subsequently, recent pre-clinical studies showed that morphine administration changed microbial composition (Banerjee et al. 2016; Wang et al. 2018). Morphine administration to rats at analgesic doses instigated a net reduction of Gram-negative Bacteroidetes and proliferation of the Gram-positive Firmicutes (Banerjee et al. 2016). Increased abundance of bacterial families belonging to phylum Firmicutes, such as *Enterococcaceae, Staphylococcaceae, Bacillaceae, Streptococcaceae* and *Erysipelotrichaceae,* were observed in morphine-implanted animals, compared to non-treated, demonstrating the potential of morphine to influence gut microbial composition (Banerjee et al. 2016). Bacteroidetes and Firmicutes are the predominant phyla comprising approximately 90% of bacterial species in the human large intestine (Cani and Delzenne 2011). Although the implications of the Bacteroidetes/Firmicutes ratio remain unclear, low ratios have been reported in people with obesity (Chakraborti 2015) and are associated with inflammatory conditions as well as metabolic endotoxemia (Boulange et al. 2016).

Although A1 beta-casein in milk can prolong GI transit via similar mechanism of action to morphine, there is currently no direct evidence to show the effects of beta-casein protein variants on microbial composition itself (Becker et al. 1990; Defilippi et al. 1995; Barnett et al. 2014). However, there is some evidence for an effect of beta-casein variants on the production of SCFAs in the gut. SCFAs are organic acids with 1–6 carbon atoms, produced during bacterial fermentation of non-digestible polysaccharides, oligosaccharides, protein and peptides in the colon (Wong et al. 2006). Acetate, propionate and butyrate are the principal SCFAs and are considered to have important physiological functions such as being an energy source for colonocytes, acting as immune signalling factors, inflammation modulators and vasodilators (Tremaroli and Backhed 2012). The randomised controlled trial led by Jianqin et al. (2016) showed that consumption of milk containing both A1 and A2 beta-casein resulted in lower concentrations of acetic acid, butanoic acid and total SCFAs in faecal matter compared to consumption of milk containing only A2 beta-casein, in addition to increasing the GI transit time. Since SCFAs are primary metabolites of gut microbiota and are considered potential indicators of events happenings in the distal colon (Macfarlane and Macfarlane 2012), it is reasonable to hypothesise microbial composition changes, however, these were not measured in this study. Also, altered concentrations of specific SCFAs or diminished concentration of SCFAs in the faeces may be associated with inflammatory conditions in the gut (Tedelind et al. 2007; Vinolo et al. 2011).

Gut inflammation and gut epithelial integrity

Evidence based on animal and human studies has shown that ingestion of A1 beta-casein protein alone or consuming milk containing the A1 beta-casein variant may be accompanied by increased GI markers of inflammation. A murine study investigated the effects of beta-casein variants on GI measures by feeding mice either A1/A1, A1/A2 or A2/A2 beta-casein variants extracted from milk for 30 days at a dose of 85 mg/animal/d. This study revealed a significant increase in inflammatory cytokines (MCP-1, IL-4), Myeloperoxidase (MPO) and antibodies (total IgE, IgG, IgG1 and IgG2a) in intestinal fluids of mice fed with the A1-like variants (A1/A1 and A1/A2). Also, the mice fed the A1-like variants demonstrated higher levels of toll-like receptors (TLR-2/4) and leukocyte infiltration in the intestine (Haq, Kapila, Sharma, et al. 2014). A similar experimental model was replicated with synthetic BCMs, feeding mice BCM-5/7 and reported increased markers of inflammation (MPO, MCP-1 and IL-4) antibodies (total IgE, IgG, IgG1 and IgG2a) and increased expression of TLR-2/4 in intestinal fluids (Haq, Kapila, and Saliganti 2014). Although these studies showed that A1 beta-casein variant and its metabolites triggered inflammation, none of these studies examined the potential of naloxone to reverse the inflammatory response in order to affirm that these were mediated via μ-opioid signalling pathways. In addition, an *in vitro* study showed that the opioid peptides released from the hydrolytic digestion of casein and gliadin reduced glutathione concentrations (a primary free radical scavenger) in neuronal cell cultures by modulating cysteine uptake (Trivedi et al. 2015). A reduction in glutathione concentrations in cells may lead to disequilibrium of cellular redox balance and restrict the anti-oxidant capacity and thereby further induce inflammation.

In humans, only one randomised controlled trial has shown a significant impact of A1 beta-casein on blood inflammatory markers (Jianqin et al. 2016). This trial showed that subjects who consumed milk containing both beta-casein variants (A1 and A2) had higher concentrations of plasma inflammatory markers (IL-4, IgG, IgE and IgG1) and BCM-7 compared to the subjects who consumed milk with the A2 beta-casein alone (Jianqin et al. 2016). Another trial that assessed the effects of the A1 and A2 beta-casein

variants on GI symptomology reported no significant differences in faecal calprotectin (non-invasive marker of GI inflammation) levels between the group consumed milk containing both A1 and A2 beta-casein and the group that consumed milk with only A2 beta-casein (Ho et al. 2014).

Interplay between gut inflammation and gut epithelial integrity

The gut epithelium is an effective physical barrier that protects the gut from pathogen invasion and is a critical element in food digestion and nutrient absorption (Kraehenbuhl et al. 1997). The paracellular spaces contain tight junctions that control the passage of fluids, nutrients and other micro-organisms from the intestinal lumen to the lamina propria (Hollander 1999). Tight junctions are composed of transmembrane proteins (e.g. occludin) peripheral membrane proteins (e.g. Zonula occludens 1 (ZO1)) and regulatory molecules (Turner 2009). Immune cells (particularly dendritic cells) and cytokines play a major role in controlling permeability of tight junctions (Clayburgh et al. 2004; Rao 2009). Many pathophysiological conditions including inflammatory diseases (Clayburgh et al. 2004), pathogen invasion (Yuhan et al. 1997) and endotoxemia (Rao 2009) have been linked to dysfunction of tight junction proteins.

Recent findings suggest the role of μ-opioid signalling pathways in gut barrier disruption. Crohn's disease is characterised as a chronic inflammatory bowel condition and dysfunction of tight junctions in the gut epithelium is a prominent feature (Schulzke et al. 2009). Smith et al. (2011) showed that administration of naloxone improved mucosal damage in Crohn's disease in humans, suggesting that opioid signalling pathways may play a role in gut barrier disruption and the resulting inflammatory process. In addition, pre-clinical evidence showed that activation of μ-opioid receptors influenced gut barrier functions. When mice were treated with morphine, mice displayed disrupted localisation of occludin and disorganised paracellular tight junction protein ZO-1 while leaving the expression of occludin and ZO-1unaffected (Meng et al. 2013). Interestingly, this study showed an increased level of TLR-2 and TLR-4 expression in the mouse intestine secondary to morphine treatment. However, tight junction protein disruption was not observed in morphine-treated, TLR knock-out mice, suggesting the contribution of TLRs in initiating gut barrier disruption. This study also confirmed an increased bacterial translocation to the mesenteric node and liver following morphine treatment, implying a potentially disruptive role of the opiate system in gut barrier function.

In addition, it has been demonstrated that morphine is linked to augmenting the secretion of a particular type of IL *in vitro* (IL-4) (Roy et al. 2005) that is associated with paracellular permeability (Capaldo and Nusrat 2009). Roy et al. (2005) showed that morphine increased the production of IL-4 *in vitro* and naloxone attenuated the effect of morphine, thus implying the involvement of μ-opioid receptor in this process. In a model of intestinal epithelial T84 cells, increased IL-4 caused barrier disruption (Colgan et al. 1994) and increased synthesis of IL-4 was observed in allergy conditions that are associated with compromised barrier function (Berin et al. 1999).

Concordantly, A1 beta-casein and BCM-7 tend to increase expression of TLR-2/4 and IL-4 (Haq, Kapila, and Saliganti 2014), molecules that are related to impaired gut epithelial integrity, however, the consequences of increased expression of these molecules in relation to gut epithelial integrity have not been investigated (Haq, Kapila, and Saliganti 2014).

Bile acid homeostasis

The evidence for the involvement of μ-opioid receptor signalling pathways in dysregulating bile acid homeostasis exists in animals. Banerjee et al. (2016) showed that morphine administration can alter bile acid pathways. Mice treated with morphine secreted abnormal levels of faecal bile acids with lesser primary and secondary bile acids than the non-morphine treated animals. Given that BCM-7 is a morphine agonist, we hypothesise that altering bile acid pathways could be one of the potential effects of BCM-7. Evidence based on epidemiological and pre-clinical studies supports this hypothesis.

Interplay between bile acid dysregulation and atherosclerosis

The major mechanism controlling plasma cholesterol levels includes conversion of cholesterol to bile acids and its subsequent excretion (Overturf et al. 1990). Dysregulation of bile acid pathways leads to excess accumulation of cholesterol and may result in atherosclerosis (deposition of plasma lipids, especially cholesterol in arterial walls in conjunction with cells and tissues) (Garcia and Khang-Loon 1996; Makishima 2005); thus, disequilibrium in bile acid pathways may contribute to the aetiology of atherosclerosis.

Epidemiological evidence suggests that consuming milk containing A1 beta-casein is associated with

coronary heart diseases (Laugesen and Elliott 2003; Bell et al. 2006). Additionally, animal studies have directly shown that A1 beta-casein accelerates the development of hypercholesterolaemia and atherosclerosis (McLachlan 2001; Kamiński et al. 2007). Feeding rabbits with A1 beta-casein significantly increased the percent surface area of the aorta covered by fatty streaks and fatty streak lesions in the aortic arch (Tailford et al. 2003). However, a mechanistic pathway by which the A1 beta-casein variant may be atherogenic was not elucidated by these studies. But, some evidence shows that A1 beta-casein may be atherogenic due to the potential of BCM-7 to catalyse the oxidation of low-density-lipoprotein (Torreilles and Guerin 1995; Steinerova et al. 2001).

The potential impact of milk opioid peptides on fractures

Opioid peptides synthesised from A1 beta-casein might influence fractures by two mechanistic pathways by: (1) triggering inflammation; and (2) binding to μ-opioid receptors in bone.

Interplay between inflammation and bone

In general, inflammation is noxious to bone (Schulte 2004) and elevated levels of inflammatory markers are associated with increased risk for fractures (Pasco et al. 2006; Dahl et al. 2015). Inflammatory bowel disease, accompanied by impaired gut epithelial integrity and inflammation, is often comorbid with low bone mineral density and high fracture risk (Schulte 2004). Furthermore, exposure to statins, which have anti-inflammatory effects, was reported to reduce the risk of fractures (Pasco et al. 2002). Inflammation triggers bone loss by accelerating osteoclastic bone resorption, augmenting bone fragility and the propensity for fractures (Hardy and Cooper 2009).

Higher milk intakes are often recommended in adults aged $\geq$ 50 to prevent osteoporosis. However, some observational evidence suggests that increased milk consumption is associated with increased risk and prevalence for fractures, although the components in milk potentially accountable for this elevated risk have not been identified (Michaelsson et al. 2014; Hilliard 2016). One plausible explanation could be related to the pro-inflammatory effects of A1 beta-casein protein fraction in milk (Haq, Kapila, Sharma, et al. 2014; Trivedi et al. 2015; Jianqin et al. 2016). The overall evidence currently available is suggestive of a weak positive association between dairy consumption and inflammation, although it is by no means conclusive (Labonte et al. 2014; Gadotti et al. 2018). Therefore, at this stage it may be postulated that the propensity for fractures increases in higher milk consumers at least partly due to the pro-inflammatory effects of A1 beta-casein; however, far more research is needed to confirm this.

Opioids in bone metabolism

There is much that remains unknown regarding the role of opioid pathways in bone regulatory mechanisms; however, there is some evidence for the presence of opioid receptors on bone cells. Perez-Casrtillon et al. (2000) demonstrated the presence of opioid receptors in human osteoblast cell line MG-63 by immunohistochemistry and RT-PCR techniques. This study indicated that μ-opioid receptor agonists, morphine and enkephalin, alter the secretion of osteocalcin in the osteoblastic cell lines. Osteocalcin production was reduced when treated with higher concentrations of these opioids. Naloxone reversed the effect of morphine-induced osteocalcin inhibition, suggesting the involvement of μ-opioid receptors in bone modulatory mechanisms (Perez-Castrillon et al. 2000). Osteocalcin serves as a biomarker for osteoblastic activity, thus reduced levels suggest reduced bone formation consistent with the pathogenesis of bone fragility. Also, administration of H-Dmt-Tic-Lys-NH-CH_2-Ph (MZ-2), a potent μ-/δ-opioid receptor antagonist (comparable to naloxone) enhanced bone mineral density in obese mice (Marczak et al. 2009). Moreover, at a 30 μM dosage, it increased the mineralisation of osteoblastic cells (MG-63) in culture (Marczak et al. 2009). In addition to these studies, a previous review concluded that opioid intake is associated with fracture risk, especially in elderly people, suggesting an involvement of opioid receptor signalling pathways in bone metabolism (Mattia et al. 2012).

Whilst there is little evidence yet to show that milk opioids can alter bone metabolism, a study conducted in a Swedish mammography cohort of 90,303 women suggests that components in milk can influence fractures. In this study, women who consumed three or more glasses of milk a day had a higher hazard ratio for any fractures compared to women who consumed less than one glass of milk a day (Michaelsson et al. 2014). The authors of this study hypothesised that the suggested detrimental effects might be driven by the D-galactose content in milk as the study showed a positive association between milk consumption,

markers of oxidative stress (urine 8-iso-PGF2) and inflammatory markers (serum IL-6). D-galactose is known to induce oxidative stress. However, this study also investigated the association between fermented dairy consumption and risk of fractures and reported an inverse association between fermented dairy consumption and fracture risk (Michaelsson et al. 2014). If the D-galactose content in dairy were to mediate inflammation and oxidative stress, fermented dairy product consumption should be positively associated with fracture risk given the higher concentration of D-galactose in fermented dairy (Alm 1982; Abrahamson 2015). Attributing the positive association between milk consumption and fracture risk to the opioid peptides produced from A1 beta-casein from milk is also plausible. Sweden is one of the countries that reports the highest A1 beta-casein consumption per capita (Laugesen and Elliott 2003). Therefore, the higher exposure to opioid peptides yielded by milk consumption in this particular geographical region may be partly mediating these effects. However, given the limited evidence available on this contention, firm conclusions on the potential of milk-derived opioid peptides to affect bone metabolism cannot be made.

The potential impact of milk opioid peptides on obesity

The association between milk consumption and obesity is inconsistent and the components in milk that may affect body weight remain understudied. Barba et al. (2005) showed an inverse association between milk consumption and body mass index (BMI) in children. Similarly, an inverse association between milk consumption and BMI was found in men and younger women in a cross-sectional study (Marques-Vidal et al. 2006). These findings are concordant with studies that have shown that increased dietary calcium intake prevents lipogenesis in adipocytes and counteracts weight gain (Zemel et al. 2000; Xue et al. 2001; Zemel 2002). Since milk consumption is a major source of calcium, this may explain the inverse association observed between increased milk consumption and obesity. Conversely, a meta-analysis of randomised controlled trials did not identify increased dairy consumption as beneficial for reduction in body weight and fat loss (Chen et al. 2012). In overweight adolescents, higher intakes of skim milk, casein and whey increased the BMI-for-age-Z-scores (Arnberg et al. 2012) suggesting a need to investigate the role of milk opioid peptides in obesity.

Interplay between opioids and obesity

The role of opioid receptors pertinent to feeding behaviours and obesity has been widely explored in animal models. Stimulation of μ-opioid receptors increases the intake of high-fat foods (Table 2). Moreover, opioid agonists are shown to induce feeding while opioid antagonists have shown to subdue feeding and mitigate weight gain (Gosnell and Levine 2009; Ziauddeen et al. 2013). Also, rats who were susceptible to high-fat diet induced obesity (Osborne-Mendel rats) expressed increased numbers of μ-opioid receptors in arcuate nucleus compared to mice who were resistant to high-fat diet induced obesity (S5B/PI rats). Administration of μ-opioid receptor agonist (DAMGO-enkephalin) in both Osborne-Mendel rats and S5B/PI rats significantly increased the preference for high-fat foods (Barnes et al. 2006). Conversely, blockage of μ-opioid receptors in the nucleus accumbens core or shell by an irreversible receptor antagonist (β-funeltrexamine) in rats attenuated the intake of palatable diet, body weight gain and fat accretion (Lenard et al. 2010). Taken together, it appears that activation of μ-opioid receptors might influence obesity partly by stimulating the preference for high-fat diet and overeating.

There are several reasons to suspect that the A1 beta-casein fraction in milk may theoretically influence obesity partly via μ-opioid receptor pathways: (1) production of μ-opioid receptor agonists during digestion; (2) the potential of these μ-opioid receptors agonist to enter circulation (Deth et al. 2016; JanssenDuijghuijsen et al. 2016; Jianqin et al. 2016); and their possible influence on the brain by crossing the blood brain barrier (Sun and Cade 2003). Therefore, regulation of body fat might be a potential function of the milk opioid peptides yielded during the digestion; however, to date there is limited evidence showing the influence of casein protein variants and their metabolites on body fat and weight.

Conclusions

The A1/A2 hypothesis postulates that the A1 beta-casein variant in milk may drive some of the negative health outcomes associated with milk consumption. However, the data are scarce and mainly derived from either epidemiology, which cannot establish causality, or animal experiments, which may not be generalisable to humans. Reduced gut motility and inflammation are linked to BCM-7, the opioid peptide yielded by A1 beta-casein digestion. However, microbial dysbiosis, impaired gut epithelial integrity, alterations of

Table 2. Implications of morphine on gut parameters, fractures and obesity.

	Outcome measures	Study design/study model	Results
Galligan and Burks (1983)	Intestinal transit time and gastric emptying in Sprague-Dawley rats	Morphine was administered intragastrically and intracerebroventricularly	Dose-dependent decrease in transit with morphine
Patten et al. (2011)	Ileal contraction in mice, rats and guinea pigs	Intact ileal sections were electrically stimulated to contract, and morphine was used to inhibit contraction *in vitro*	Morphine inhibited illeal contraction
Runkel et al. (1993)	Bacterial translocation in rats	Following a subcutaneous administration of morphine bacterial counts were taken in mesenteric lymph node complex, blood, spleen, liver, duodenum, jejunum, ileum and caecum	Significant bacterial count increase in each intestinal segment
Banerjee et al. (2016)	Gut microbiota composition in mice	Morphine administered in analgesic dose as slow release morphine pellets	Increased abundance of bacterial families belonging to phylum Firmicutes, such as *Enterococcaceae, Staphylococcaceae, Bacillaceae, Streptococcaceae,* and *Erysipelotrichaceae*
Wang et al. (2018)	Gut microbiota composition in mice	Morphine administered via pellet implantation method	Significant shift in gut microbiome composition and induced dysbiosis showing significant increase in species with pathogenic functions
Meng et al. (2013)	Gut epithelial integrity in rats	Morphine administered via pellet implantation method	Increased level of TLR-2/4 expression in conjunction of disrupted tight junction protein expression
Banerjee et al. (2016)	Bile acid homeostasis in mice	Morphine administered via pellet implantation method	Abnormal levels of faecal bile acids with lesser primary and secondary bile acids
Perez-Castrillon et al. (2000)	Osteocalcin function in cell culture	Osteoblastic cells (*in vitro*) treated with morphine	Reduced osteocalcin secretion with higher concentration of morphine
Barnes et al. (2006)	Intake of high-fat diet in rats	Blockage of μ-opioid receptors in arcuate nucleus by DAMGO-enkephalin	Increased preference for high-fat diet

TLR-2/4: toll-like receptor-2/4; DAMGO: enkephalin-μ-opioid receptor agonist.

bile acid metabolism, increased risk for fractures and obesity are all possible but understudied potential implications of BMC-7. Given the widespread consumption of milk as a major source of protein and calcium from infancy to the elderly, it is imperative to investigate the possible effects of these opioid peptides produced from the A1 beta-casein protein variants. Adequately powered, randomised controlled trials in humans are warranted to unravel the clinical relevance of BCM-7 and A1/A2 milk consumption. The findings from such studies will inform public health messages and strategies for the prevention and management of diseases.

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