Synopsis of the thesis titled

Isolation and Characterization of Genetic Variants of Beta-Casein Protein (A1/A2) and Study Their Impact on Early Precipitation of Osteoporosis

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DOCTOR OF PHILOSOPHY

by

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1. Introduction:

Cow milk is the most vital source of proteins and microelements, which are important constituents for healthy bone. Caseins account for 80% of bovine milk protein, beta-casein proteins make up approximately 30% of the total bovine milk protein and both have great nutritional and industrial importance. Recent studies have shown that a genetic shift, causes polymorphism at codon 67 of the beta-casein gene, exon VII, chromosome no.6, lead in to the formation of different allelic variants including most common A1 and A2. As a result of this mutation, a conversion from cytosine to adenine base, leads to the replacement of proline (A2 allele, codon; CCT) by histidine (A1 allele, codon; CAT) amino acid, at position 67(**Fig.1)**. The A2 allele is recognized as the progenitor of beta-casein gene in the genus Bos, a high concentration of A2 is observed in Indian zebu cattle, A1 is most frequent in exotic cattle.

A1 variant during enzymatic hydrolysis, microbial fermentation and gastrointestinal digestion leads to the formation of powerful toxin, beta-casomorphin 7 (BCM7). BCM7, resists further hydrolysis by intestinal brush border enzymes and get absorbed intact, enters the systematic circulation, and expresses its effect, after reaching endogenous receptors. BCM7, has an opioid like structural motif, that binds to μ-opioid receptors, due to which it is implicated in many illnesses such as heart disease, Type-1 Diabetes Mellitus, Sudden Infant Death Syndrome, Schizophrenia, etc. These manifestations are mainly associated with individuals consuming milk from A1 allelic variant cows.

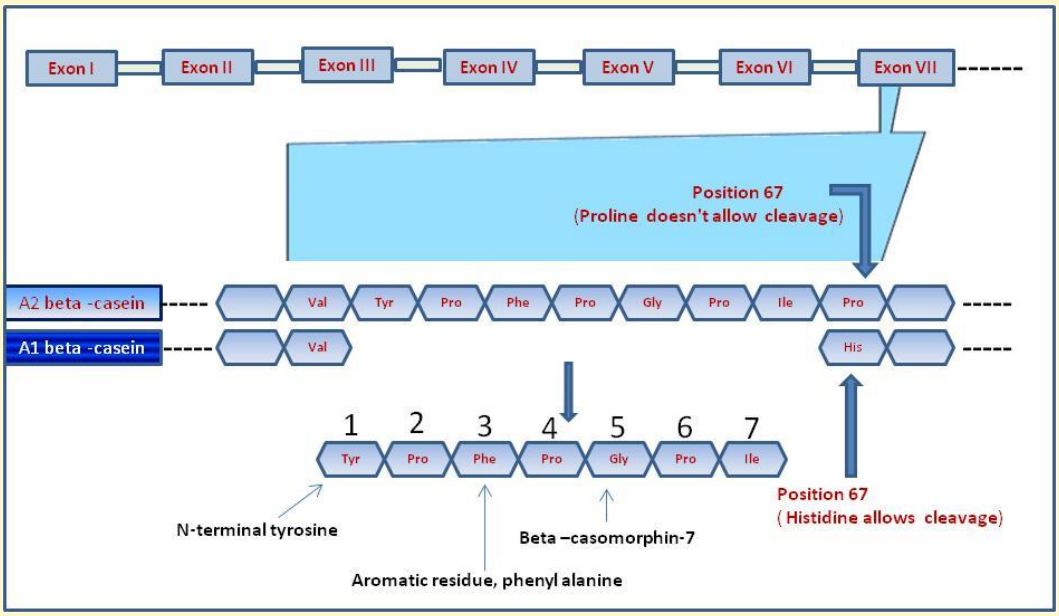

Fig. 1: Release of BCM7 and its structural similarity with opioids. Milk-derived bioactive peptides can be released by microbial fermentation or enzymatic hydrolysis. A1 and A2 beta-casein proteins showing the amino acid difference at position 67. A1 variant has one histidine amino acid at position 67 that undergoes the enzymatic cleavage of the peptide bond, and BCM7 is released. The A2 variant does not cleave due to the presence of a proline amino acid at this position.

BCM7 by directly acting on μ-opioid receptors, impairs the activity of osteoblast and also reduces bone mineral density (BMD). Indirect mechanism suggested is the suppression of hypothalamic pituitary gonadal (HPG) axis and hypothalamic pituitary adrenal (HPA) axis, where dysfunction of both, results in hypogonadism. Estrogen deficiency impairs the normal cycle, by increasing osteoclastic resorption activity, without corresponding increase in osteoblastic formation activity.

In brief, this section provides selective information on: genetic variants of beta-casein gene, production of BCM7, its structural similarity with opioids and direct/indirect effect of opioid on precipitation of osteoporosis. However, impact of opioid peptide/BCM7 in osteoporosis is not very well explored, and related information available, is either based on epidemiological data taken from humans or is related to *in vitro* studies. Hence, an

in-depth analysis is required to understand and delineate the exact role/mechanism of BCM7 in the precipitation of osteoporosis. The present study was aimed to explore the A1/A2 allelic variation in Hariana and Holstein Friesians (HF) cows, isolation/characterization of beta-casein and impact of its variants (A1/A2) on the early precipitation of osteoporosis.

Worldwide, a bone breaks due to osteoporosis every three seconds. Researchers estimated, that 1 in 3 women above the age of 50 will experience osteoporotic fractures, as well as 1 in 5 men. In Europe, India, Japan and the USA alone, there are an estimated 125 million people with osteoporosis. As 10% of the Indian population (more than 100 million) are over 50 years of age, they are on high risk to experience osteoporotic fractures. As per WHO, 2012 report, nearly 300 million people suffer from osteoporosis in India and in the next decade it may increase as high as 50 percent of the population. Another research study in Delhi, estimated the prevalence of osteoporosis as 24.6% in men and 42.5% in women, above 50 years of age. Thus considering the growing prevalence rate of osteoporosis, the proposed study on the association of osteoporosis with consumption of exotic cow milk, is observed to be significantly important.

2. Objectives:

1. To identify genotypes of HF and Hariana cows in and around Pilani (100Km).
2. To develop a technique for nonenzymatic isolation of beta-casein from milk.
3. To perform comparative analysis of osteoporosis induction in rats using surgical method and by feeding of milk protein / natural milk.
4. To analyse the correlation between consumption of beta-casein variants of milk and early precipitation of osteoporosis.

3. Methodology:

Genotype of cattle in Pilani region was estimated by sequencing the exon VII, followed by multiple alignment and chromatogram analysis. Cattle were then divided in A1A1, A1A2 and A2A2 genotypic groups. Milk samples from A1A1 cattle were collected to develop a non- enzymatic method of beta-casein protein isolation. A non-genetic animal model for osteoporosis was standardized in adult, female Wistar rats (180-200 gm body weight) by performing ovariectomy. The ovariectomized rats were used as osteoporotic animals for comparison with other study rats. The test groups were either fed with beta-casein protein isolated from A1 milk, or natural milk from A1A1 and A2A2 cows, in order to compare the effect of isolated protein and proteins present in natural milk. Synthetic BCM7 was obtained from Biolinkk (India) and given by both intraperitoneal (IP) and oral (PO) routes as aqueous solution of peptide, with dose of 1mg/kg, to compare the effects of enzymatic hydrolysis on BCM7 (during gastrointestinal digestion and absorption). Dosing for all animals **(Table 1)** was done, for 4 months along with the normal pallet diet (NPD).

Table 1: Animal groups, their dosing and feeding.

S. No.	Group name	No. of animals	Description of animals	Dosing /feeding (for 4 months)
1	Normal control	6	Natural / normal rats of same age group	NPD
2	OVX	6	Ovariectomized rats	NPD
3	Sham operated	6	Rats with incision only in skin	NPD
4	BCM7 (PO)	6	Normal rats + BCM7	BCM7 @1mg/ kg PO; + NPD
5	BCM7 (IP)	6	Normal rats + BCM7	BCM7 @1mg/ kg IP;+ NPD
6	A1 beta-casein protein	6	Normal rats + BCM7	Isolated beta-casein protein @1mg/ kg PO;+ NPD
7	A1 natural milk	6	Normal rats + A1 natural milk	A1 natural milk @ 7.8ml/ kg* (equivalent to 1mg/kg) PO;+ NPD
8	A2 natural milk	6	Normal rats + A2 natural milk	A2 natural milk @ 7.8ml//kg* (equivalent to 1mg/ml) PO;+ NPD

Dose of BCM7 was decided based on available literature (Dubynin et al., 2008; Yin et al., 2010; Zhang et al., 2012; Zoghbi et al., 2006; Zong et al., 2007). * Beta-casein in natural milk was calculated by Bradford assay of isolated protein and an equivalent volume of milk was fed.

To investigate the impact of this treatment/feeding, on precipitation of osteoporosis parameters investigated were (1) serum osteocalcin levels (2) serum calcium levels (3) serum phosphorus levels (4) histopathology of bones (5) microcomputed tomography (µCT) analysis (6) biomechanical analysis (tensile strength, yield point, energy, Young's modulus and elongation) of femur (7) change in body and uterus masses.

4. Results and discussions:

4.1: Beta-casein gene sequence analysis:

The overall genotype of HF cattle identified had 9% A1A1, 69.7% A1A2 and 21.3% A2A2 alleles. Whereas most of desi cattle were observed to possess A2A2 genotype, A1A2 was observed only in mixed zebu breeds whereas A1A1 was not observed. The allele frequency analysis of HF cows was 0.44 for A1 allele, 0.56 for A2 allele and 0.09, 0.70, 0.21 for genotypes A1A1, A1A2, and A2A2, respectively. The allele frequency analysis for Zebu cows was 0.1 for A1 allele, 0.9 for A2 allele and 0.00, 0.10, 0.9 for genotypes A1A1, A1A2, and A2A2, respectively. The distribution of the genotypes was within Hardy–Weinberg equilibrium in the tested population ($P>0.05$). The value of gene homozygosity (Ho) was observed as 0.40, gene heterozygosity (He) as 0.60 and fixation index (FIS) as - 0.20. During the study, a large proportion (69.7%) of HF cows having heterogeneous (A1A2) genotype was identified, which indicated an abrupt increase of A1 allele, as compared with desi cows. It was observed that continuous breeding of such HF cattle (A1A2 or A1A1), may replace the A2 allele from the population, in the near future. Under Indian circumstances, genotypic estimation is expected to play a major role in promoting breeding for A2 allele. New breeding policies are to be proposed to increase insemination with A2A2 semen only. Awareness among farmers about these alleles is expected to play an important role to achieve a higher proportion of A2A2 cows.

4.2: Beta-casein protein isolation:

Beta-casein was isolated by rennin and acetic acid coagulation methods, fractions isolated were analyzed using 18% SDS -polyacrylamide gel electrophoresis (PAGE). Multiple bands were observed in rennin method, indicated sub-fractions of casein or whey proteins. Similarly when the acetic acid processed sample was analyzed, three (2

intense and 1 faint) bands were observed (**Fig.2)**. Principal protein was beta-casein [(β-CN), (mol. wt. ≈25KD, upper intense band)] with minor amount of α_{s1}-casein [(α_{s1}-CN), (mol. wt. ≈20KD, lower intense band)] and alpha $_{s2}$ -casein [(α_{s2}-CN) (middle faint band)]. All the bands obtained were further analyzed using MALDI-MS/MS.

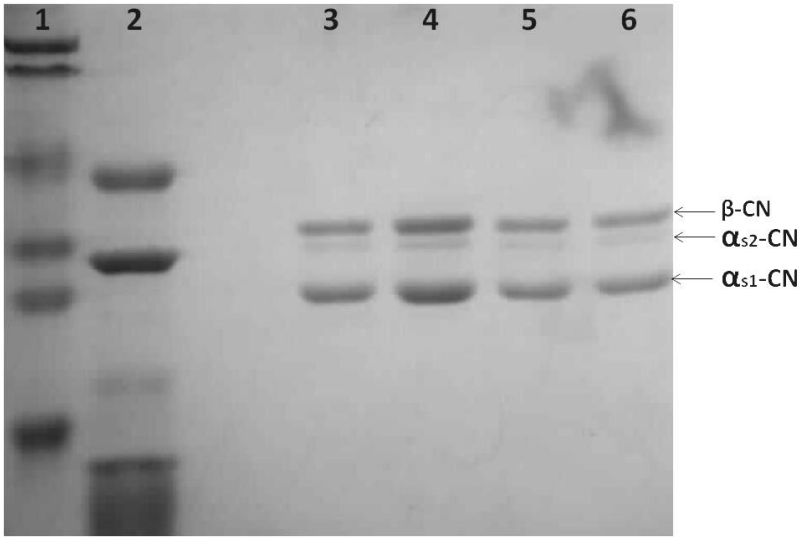

Fig. 2: SDS–PAGE electrophoretogram of supernatant obtained from the milk of different breeds. **Lane 1:** Holstein Friesians (HF) milk was coagulated by rennin and obtained rennet curd was heated at 80 ºC for 5 min, incubated at 4 ºC for 24h. All other samples were coagulated by acetic acid; obtained curd was washed and dissolved in AMQW, incubated at 4 ºC for 24h. **Lane 2**: low molecular weight marker (PMW-L), **Lane 3-6:** supernatant from milk samples of different cows.

Acetic acid method was faster, efficient and economical, as compared to the enzymatic method for isolation of beta- casein from milk. The cost for coagulation of milk using glacial acetic acid was approximately 500 times lower than use of enzymes such as rennin. Acetic acid method, was carried out at room temperature, while high temperature was required in rennin method to inactivate rennin. Thus scaling up of the method; will not add extra cost of high temperature.

4.3: Animal study:

4.3.1: Estimation of serum osteocalcin levels:

Osteocalcin is a vitamin K-dependent calcium-binding non-collagen protein, specifically produced by osteoblast, and has been used as one of the osteoblast marker. Osteocalcin is released from bone matrix into blood by action of various enzymes during bone metabolism. To estimate serum osteocalcin, Anti-Glu, specific monoclonal antibody (as described by Kit manufacturer) was used. There was a significant decline in serum osteocalcin levels of ovariectomized, BCM7 treated, beta-casein protein and A1 natural milk fed, rats. Lowest levels of osteocalcin was observed in BCM7 (IP) treated rats, while normal levels of osteocalcin was observed in sham operated and A2 natural milk fed rats. There was comparatively less decrease of osteocalcin in BCM7 (PO) treated rats, as compared with BCM7 (IP) treated rats; possibly due to the degradation of the peptide in the stomach and other parts of the gastro-intestinal tract (GIT). Thus, decrease in serum osteocalcin levels indicated, low osteoblast and high osteoclast activity or increased bone resorption.

4.3.2: Estimation of serum calcium and phosphorus:

There was a significant decrease in serum calcium and phosphorus levels in ovariectomized, BCM7 treated, beta-casein protein and A1 natural milk fed rats, as compared with normal control rats, at the end of the study. A2 natural milk fed rats exibited better levels of both the minerals as compared with normal control rats. A significant difference in serum calcium and phosphorus levels was observed after 3 months of dosing, not earlier.

It is known well that calcium and phosphorus in milk, play a pivotal role in maintaining healthy bone. Low calcium levels stimulates the secretion of parathyroid hormone (PTH), which in turn increases the precipitation of osteoporosis by triggering bone metabolism, causing bone resorption and bone loss besides releasing calcium ions in the blood. PTH also increases renal clearance of phosphorus, resulting in hypophosphatemia. Low

levels of both these minerals and osteocalcin, in BCM7 treated, beta-casein protein, and A1 natural milk fed rats, indicated unhealthy bone. However, better levels of these minerals observed in A2 milk treated rats, supported the fact that natural milk is a good source of calcium and phosphorus and its role in maintaining healthy bones.

4.3.3: Histology:

4.3.3.1: Observation of bone cells:

Observations on cells involved in bone remodeling, revealed an increase of osteoclast cells and decrease of osteoblast cells in OVX rats, BCM7 treated rats, beta-casein protein and A1 natural milk fed rats, as compared with control group rats.

4.3.3.2: Articular surface erosion:

Bone surface erosion was analyzed histologically by looking at cross section of the femoral and tibial condyles stained by Toluidine-blue. Surface erosion indicated alterations in the structure of the collagen fibers in comparison with intact cartilage surface of normal control rats. Surface erosion was more pronounced in OVX rats and clearly observed in, BCM7 treated rats, beta-casein protein and A1 natural milk fed rats. No significant changes were observed in sham operated rats and A2 natural milk fed rats.

4.3.4: Microcomputed tomography (µCT) analysis:

The representative µCT images are shown in **Fig. 3** were obtained by using µCT scans. The highest bone loss was observed in OVX rats, followed by BCM7 IP rats. Less loss was observed in BCM7 PO rats, as compared to BCM7 IP, which indicated that BCM7 was not absorbed completely. It was either degraded in GIT or passed through it.

Isolated protein fed rats had less bone loss, as compared with BCM7 treated rats, but higher than A1 natural milk fed rats.

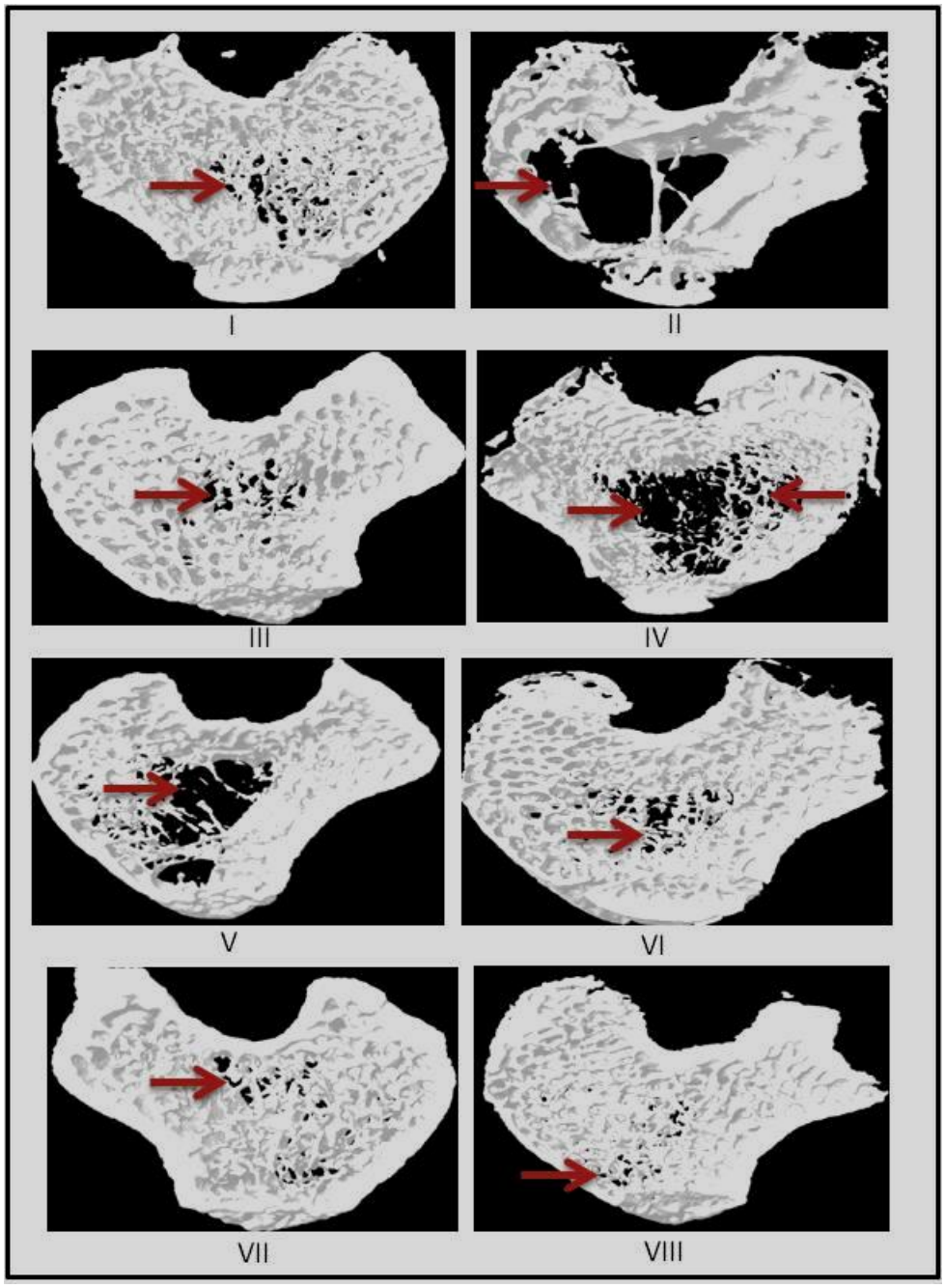

Fig. 3: Representative 3D images generated by µCT in different groups of rats at age of 6.5 months. ROI was drawn bellow the growth plate of tibia. Images acquired at 70 kV, 142mA using 1 mm filter at a resolution of 18 micro meter/ pixel. Groups of rats are: I-control, II-OVX, III-sham operated, IV-BCM7 oral, V-BCM7 I/P, VI-isolated beta-casein, VII- A1 milk, and VIII-A2 milk.

Quantification of the tarbecular bone volume fraction (BV/TV), thickness of trabecularized spicules (Tb.Th), trabecular separation (Tb.Sp) and trabecular number (Tb.N) was conducted using µCT scans. Data revealed that OVX, BCM7 treatment, A1 beta-casein and A1 natural milk feeding, significantly decreased trabecular bone volume, trabecular number, thickness of trabecularized spicules and significantly increased trabecular space, as compared with normal control rats.

4.3.5: Biomechanical analysis:

Tensile strength testing was performed using a universal testing machine (UTM) (UNITEK 94100). Both ends of the bone were fixed and tensile strength was assessed, operating the equipment, at a speed of 1 mm/min, until the bones were fractured. The data extrapolated from the graphs (load-displacement and stress-strain) as obtained from UTM, showed a clear difference in strength of bones for different groups. Maximum tensile strength, yielding point, energy and Young's modulus were recorded for A2 natural milk fed rats, while minimum for OVX rats. Significant difference in all the mechanical parameters was observed in all treatment groups, as compared with control group rats. Significant decrease in tensile strength, yielding point, total energy absorbed and Young's modulus was observed in BCM7 IP, isolated protein and A1 natural milk fed rats, as compared with BCM7 PO treated rats. Significant increase in mechanical parameters was observed in A2 natural milk fed rats, as compared to A1 natural milk fed rats, indicating good effect of A2 natural milk on bone health. There was significant decrease in elongation capacity of bone in all OVX and treated rats.

4.3.6 Analysis of body and uterus masses:

Hypogonadism impairs the normal body metabolism, by lowering the level of estrogen and testosterone, resulting in increased body mass, in all females and males, respectively. Significant increase in body mass was observed in OVX, BCM7, beta-casein protein, A1 and A2 natural milk treated rats, as compared with control rats. Mass gain in control rats at termination of study (6.5 months) was 34% and in OVX rats, 38%. Ovariectomy induced tremendous regression of the uterus in OVX rats, there was a significant decrease in uterus mass in BCM7 (IP), beta-casein protein and A1natural milk treated rats.

The probable mechanism involved in indirect effect of BCM7, that suggests precipitation of osteoporosis is the suppression of HPG axis, by action of BCM7 on μ-opioid receptors. Normally, gonadotropin-releasing hormone (GnRH), released by hypothalamus, activates the anterior pituitary gland to release luteinizing hormone (LH) and follicle stimulating hormone (FSH). These hormones, via systemic circulation, exhibit their effect on testes and ovaries, to produce testosterone or estrogen, respectively. The structural similarity of BCM7 with opioids, indicate their possibility to either act on μ-opioid receptors, suppressing the HPG axis or on the ovaries and testes, leading to the reduction of estrogen and testicular testosterone production respectively, resulting in the precipitation of osteoporosis, as similar to that of opioids. The HPA axis is another endocrine system that is affected by the peptide.

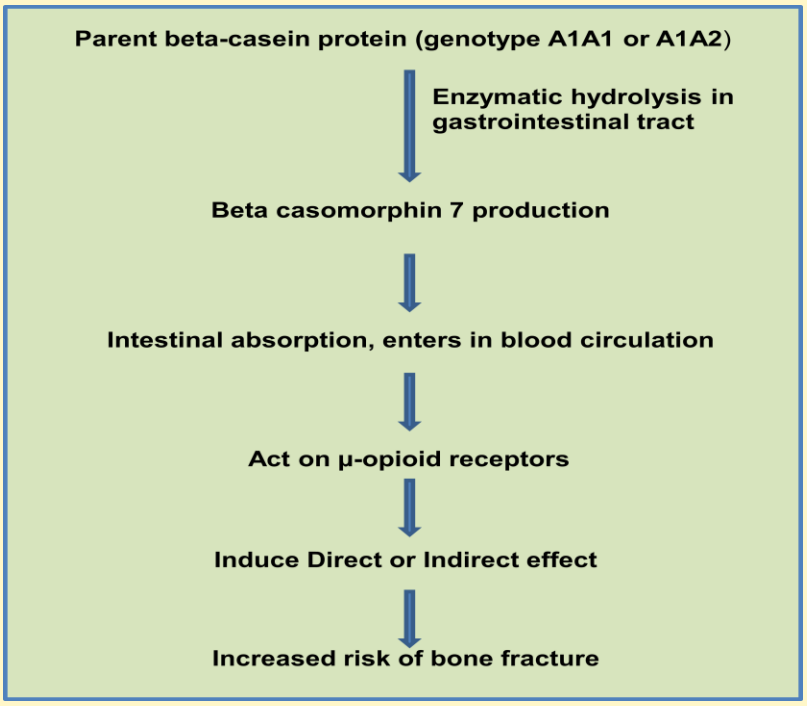

Fig. 6.1: Flow chart depicting suggestive pathway of BCM7, in increasing risk of bone fracture.

5. Conclusions:

Highest degradation of bone and decrease in tensile strength was observed in BCM7, A1 milk protein and A1 natural milk treated rats, indicated the weakening of rat bones and supports the proposed hypothesis. Articular surface erosion and increase in osteoclast cells in these rats, strongly indicated the precipitation of osteoporosis. Low levels of osteocalcin, suggested that BCM7, that acts on μ-opioid receptor of osteoblast cells, induced hypogonadism by suppressing HPG and HPA axes, resulting in bone resorption and decreased bone formation. Rats, in spite of consuming natural A1 milk, expressed low levels of calcium & phosphorus, leading to decreased tensile strength of the bone, clearly indicating the adverse effect of BCM7, on bone formation and strength.

Hence, based on these aforementioned studies, it may be inferred that people consuming milk of A1 variant cattle on a regular basis, may be at high risk of osteoporosis, or its early precipitation. Further specific studies, in a larger population and human trials, may be of importance to strengthen the findings and the hypothesis.

6. Future scope of the work:

- Development of a kit for identification of A1 or A2 cattle at the spot.
- Assessment of Genotypes, A1 and A2 milk producing cattle will help play a major role in promoting breeding of A2 allele type and lower exposure to A1 allele type, with regulated/proper breeding plan. Awareness among farmers about these alleles would help achieve higher proportion of A2A2 cows.
- Reconfirming the findings for precipitation of osteoporosis in rats, through a clinical study on the human population.