Vitamin B_{12} as a regulator of bone health

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In the early 1920s, the anti-anaemic effect of liver-rich diet had been recognized. The anti-anaemic substance from the liver was isolated by 1950 and called 'vitamin B_{12}' (hereafter B_{12}). It took another 20 years to structurally define and chemically synthesize B_{12} in its pure form. Since then, it has been recognized that B_{12} modulates a variety of biological systems, from immune system to bone homeostasis. Recent clinical studies have shown that B_{12} deficiency is likely to be an important etiological factor in the pathogenesis of bone degeneration. In this regard, either observational studies that aimed to verify an association between low B_{12} level and bone mass, or clinical trials on the effect of B_{12} as a supplementary treatment in low bone mass patients have been presented in the emerging clinical literature. Recently, we created a mouse genetic model of B_{12} deficiency to elucidate its mode of action by genetic deletion of gastric intrinsic factor, a protein essential for the absorption of B_{12} from the gut. This has led to the identification of a novel gut–liver-bone axis that has the potential to be pharmacologically targeted for treating low bone mass diseases in humans. In this review, we revisit the history of B_{12} from its discovery in the early 20th century to the elucidation of its mode of action in the bone till date.

Keywords: Bone formation, endocrinology, osteoblasts, vitamin B_{12}.

BONE growth during gestation and post-natally is regulated by the process of bone remodelling[1]. Bone formation carried out by osteoblasts and resorption carried out by osteoclasts form the two arms of this remodelling process, which ensures that vertebrates have flexible yet strong skeleton during most part of their adult life. A dysregulation in either arms of the remodelling process, formation or resorption, leads to low bone mass and increased risk of fractures[2]. In the last 20 years advances in mouse genetics and studies in clinics have identified numerous factors that regulate bone mass. The factors that affect bone health originate either within the bones or from other organs. For instance, serotonin that originates from the gut acts as a hormone to regulate bone formation by acting on osteoblasts, while bone morphogenetic proteins originate within the bone marrow and affect bone formation[3]. Despite the advances in mouse and human molecular genetics, our search for an ideal therapy that can cure low bone mass diseases continues.

Vitamins are (semi)essential nutrients required for the proper functioning of the human-body systems[4]. Vitamins perform diverse functions by acting as hormones or local growth factors to impinge on critical biological processes within different cell types, viz. vitamin D_3 regulates bone forming activity of the osteoblasts in the bone. Multiple vitamins have been shown to regulate bone mass and their mechanisms of action have been well established, viz. vitamins D, K and E[5]. In our quest to identify biological factors that regulate bone mass, we examined the role of vitamin B_{12} (hereafter B_{12}) in regulating bone mass[6]. Before going into details of this study, it would be appropriate to go through the historical investigations on this complex vitamin that have lasted for more than a century and led to the award of two Nobel Prizes.

Discovery of B_{12}: a long dwindling road

Pernicious anaemia (PA) is a condition in which the blood has lower than normal number of red blood cells[7]. PA was first described by Thomas Addison in 1849, and was generally referred to as Addison's anaemia, a disease with an unknown cause leading to death of affected persons (hence termed pernicious). Up until 1920, there was no cure for Addison's anaemia. In 1923, Minot and Murphy[8] were the first to test the radical concept that dietary intake of raw liver might help patients with PA. Their idea of feeding patients with liver was based on earlier experimental work by George Hoyt Whipple with a canine experimental model of anaemia. In this model, Robscheit-Robbins and Whipple[9] showed that amongst the foods tested, meat products (especially liver) were highly beneficial in reversing anaemia (http://www.bloodjournal.org/content/bloodjournal/suppl/2006/06/12/107.12.4970.DC1/Video1.mov). Similar to the findings in the canine model, feeding PA patients with raw liver cured their disease. This discovery led to Minot, Murphy and Whipple sharing the Nobel Prize in Physiology or Medicine in 1934. The work of Minot and Murphy led to the conclusion that an 'extrinsic factor' was responsible for this remarkable effect of liver on the health of PA patients[10,11]. In 1948, two teams, one directed by Karl Folkers (Merck & Co., Inc.), and the other by Alexander R. Todd (Glaxo laboratories), obtained anti-pernicious or extrinsic factor itself and called it 'vitamin B_{12}'[12]. Up until this point the only way one could isolate B_{12} was to use

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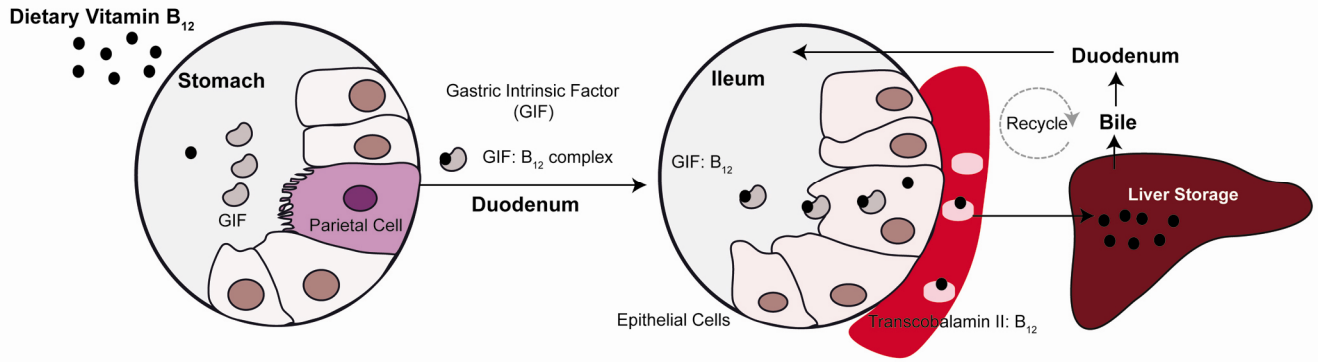

Figure 1. Vitamin B_{12} physiology in animals. Vitamin B_{12} is obtained from a diet rich in animal products, binds to gastric intrinsic factor (Gif) in the duodenum and is absorbed through receptor-mediated endocytosis in the ileum. In the blood, it binds to transcobalamin II protein and is transported to the liver, where in it is stored and recycled.

litres of cultures of B_{12} synthesizing bacteria or tonnes of liver extracts and laborious chromatographic methods. The difficulties in B_{12} isolation necessitated the need to chemically synthesize it in pure form so that it is safe for clinical use. To facilitate the elucidation of chemical structure, Merck provided a small amount of B_{12} powder to Dorothy Hodgkin[13], a well-known British scientist who described the chemical structure of B_{12} in 1958. With the elucidation of the structure of B_{12} by Hodgkin, chemists now had the information to attempt synthesis of B_{12} (http://www.webofstories.com/play/dorothy.hodgkin/35). Robert B. Woodward (Harvard University, USA) in close collaboration with Albert Eschenmoser (ETH, Switzerland) took up the challenge to synthesize B_{12} (refs 14, 15). It took Woodward and Eschenmoser a decade to chemically synthesize B_{12}; the final announcement of its complete synthesis came in the fall of 1972 (http://www.chem.umn.edu/groups/hoye/links/condensedwoodward.php). Thus, it took the efforts of biologists, clinicians and the industry almost 50 years to identify, structurally define and chemically synthesize B_{12}, the very deficiency of which leads to PA.

B_{12} physiology in animals

In the animal kingdom only protozoans and certain bacterial species can synthesize B_{12}, and during evolution organisms lost the ability to synthesize B_{12}, but it remained essential for their survival[16]. In the intestinal tract, diet-derived B_{12} binds to gastric intrinsic factor (Gif) produced by the parietal cells of the stomach (Figure 1)[17]. The Gif–B_{12} complex is recognized by the Cubilin receptor assembly in the small intestine, leading to its endocytosis. B_{12} is next transported to the blood where it binds to the protein transcobalamin II (Tcn2). Following this, Tcn2–B_{12} complex is transported to the liver, where B_{12} is released. This pool of B_{12} serves as a store to provide a continuous supply of the vitamin or its derivatives to other parts of the body[16–19]. Within the cells, B_{12} is converted into two known cofactors, methyl-B_{12} and 5′-adenosyl-B_{12} (Figure 2)[16–19]. Methyl-B_{12} is essential for the functioning of methionine synthase, an enzyme required for the production of methionine in the animal cells. Methionine in turn is responsible for the generation of S-adenosyl methionine or is incorporated into proteins. 5′-adenosyl-B_{12} on the other hand, is essential for the functioning of the enzyme methyl malonyl CoA mutase that generates succinate. However, methionine can be derived from the diet and succinate can be generated as a by-product of the Krebs cycle. Studies are needed to identify other factors that are produced by B_{12}-dependent reactions to understand the importance of B_{12} in the cells.

B_{12} deficiency: genetic and induced causes

The historical perspective provided above should not give the view that B_{12} deficiency is only associated with PA. Anaemia is only one of the symptoms of B_{12} deficiency, which has multiple causes, can happen at any stage of a person's life from the time he/she is growing in his/her mother's womb to adult life, and can lead to multiple organ dysfunctions[20–23]. In clinical practice, diagnosis of B_{12} deficiency is typically established through the measurement of serum cobalamin (Cbl) levels, and a person is defined as deficient when B_{12} serum levels fall below 200 pg/ml. However, given that most of the B_{12} is stored in the liver and is not circulating functional B_{12} deficiency can occur at any serum level, with or without anaemia and/or macrocytosis[20]. B_{12} deficiency can be reflected in elevated methylmalonic acid (MMA) and homocysteine (Hcy) levels, but these two molecules are not routinely tested unless the initial B_{12} levels are equivocal, because MMA and Hcy can be elevated in other conditions as well that are independent of B_{12} levels. B_{12} deficiency is common in all age groups and is sometimes diagnosed late due to the lack of a reliable assay and its complex etiology[19,20].

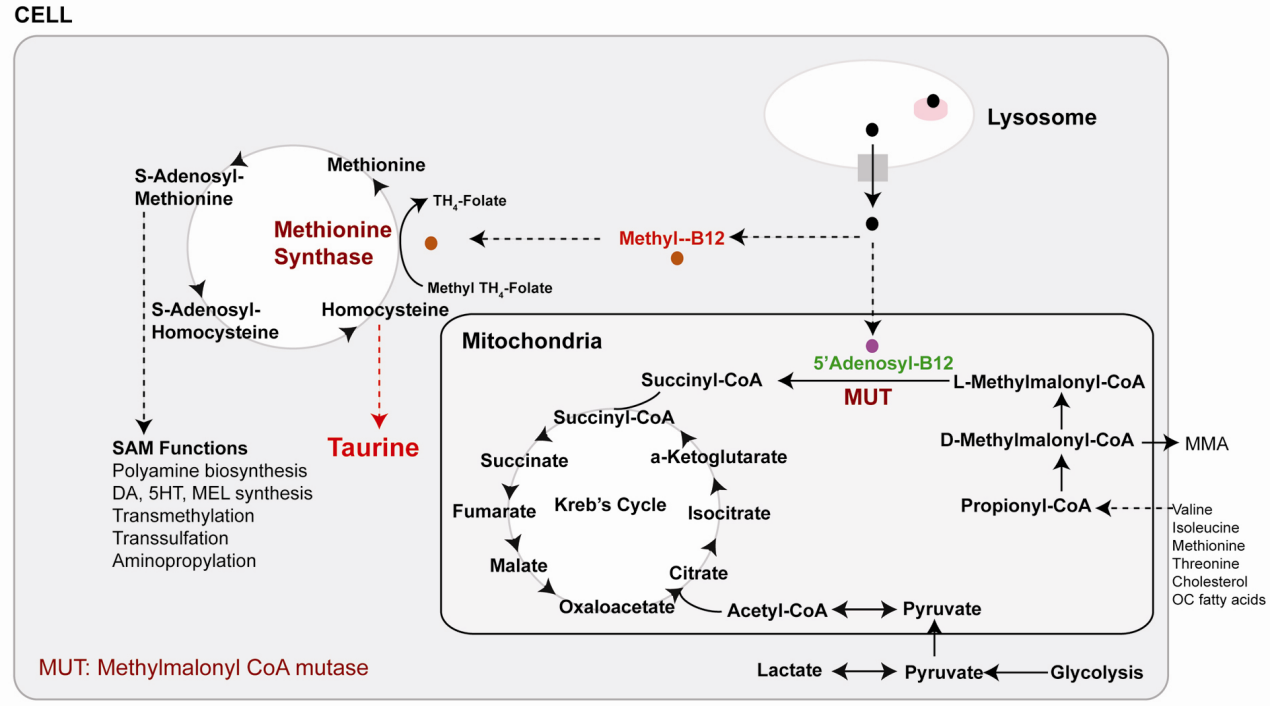

Figure 2. B_{12}-dependent enzyme reactions and associated metabolic processes. Vitamin B_{12} is taken up by the cells through the process of endocytosis and then through a series of enzymatic reactions is converted into two cofactors, methyl B_{12} and 5′ adensyl B_{12}. The B_{12} derived cofactors are essential for two known enzymatic reactions, methionine synthase and methylmalonyl CoA mutase, and through their downstream products affect a variety of biological processes.

During gestation, placental transfer of B_{12} from the mother provides adequate source to the foetus that lasts up to 1 year after birth[21]. A deficiency of B_{12} in the mother due to inadequate B_{12} source in the food (pure vegetarian diet), therefore leads to various abnormalities in the offspring during gestation or after birth. In adults, B_{12} deficiency is generally a result of certain conditions that affect stomach Gif synthesis, such as atrophic gastritis, in which the person's stomach lining has undergone thinning; gastric bypass surgery that removes part of the stomach or small intestine, including weight loss surgery; conditions that affect the small intestine where B_{12} is absorbed, such as Crohn's disease, celiac disease, bacterial growth, or a parasite; heavy alcohol consumption, and immune system disorders, such as Graves' disease. B_{12} deficiency is more common in persons who do not eat any animal products (including meat, milk, cheese and eggs), or persons who do not eat enough eggs or dairy products to meet B_{12} needs[24].

Changes in B_{12} levels and bone health in humans

In humans, changes in serum B_{12} levels have been reported to affect growth, bone mineral density and bone fracture risk[24–28]. The effect of B_{12} deficiency or supplementation on bone is site-specific and dependent on a variety of factors, including age, gender and duration of deficiency/treatment.

B_{12} deficiency has been shown to lead to a decrease or no change in bone mineral density (BMD). Framingham Osteoporosis Study identified that in both men and women B_{12} deficiency was associated with lower BMD[27]. In men, significant effect was observed at the hip, while in women at the spine. Dhonukshe-Rutten[29] examined 1267 subjects of the Amsterdam Longitudinal Aging Study (subjects were 615 men and 652 women with a mean age of 76 ± 6.6 years) for changes in B_{12} levels, bone biomarkers and fracture risk. This study showed that low B_{12} levels (<200 pM) were significantly associated with markers of bone turnover, and an increased risk of fractures. Cagnacci *et al.*[30] studied 117 healthy postmenopausal women, at two time-points five years apart who volunteered for a cross-sectional evaluation of BMD and levels of serum folates, homocysteine and B_{12}. Their goal was to determine whether in postmenopausal women, levels of folates, homocysteine or B_{12} could predict changes in vertebral BMD. Their study could not find any significant corelation in vertebral BMD and homocysteine or B_{12} in healthy women immediately after menopause. Morris *et al.*[31] studied vitamin status indicators and bone health on data collected from older (i.e. aged >55 years) men and women who underwent DEXA scans of the hip as participants in the US National Health and Nutrition Examination Survey ($n = 1550$). This analysis showed that in older Americans serum B_{12} levels were positively associated with BMD in a dose-dependent fashion up to ~200 pmol/l.

In contrast to the negative effects of B_{12} deficiency on bone density, data on B_{12} supplementation have not been equivocal. The effect of B_{12} supplementation is dependent on age and other factors. Rejnamrk *et al.*[32] examined whether dietary intake of B_{12} (as assessed by food records) affects BMD and fracture risk. They utilized a population-based cohort, including 1869 peri-menopausal women from the Danish Osteoporosis Prevention Study, and examined any possible associations between B_{12} intake and BMD at baseline and after five years of follow-up. They did not find any association between changes in dietary intake of B_{12} and BMD. Although supplementation with B_{12} alone has not been found to affect fracture risk, but in combination with other molecules B_{12} has shown either a positive or no effect. Herrmann *et al.*[33] tested the effect of supplementation of vitamin D_3 in combination with B_{12} but could not find any further improvement in bone biomarkers in these subjects (>54 years). In contrast, combinatorial supplementation of healthy subjects with B_{12} and folate showed a positive effect. Sato *et al.*[34] tested whether treatment with folate and B_{12} reduced the incidence of hip fractures in patients (>65 years) with hemiplegia following stroke. The study showed that in this Japanese population with a high baseline fracture risk, combined treatment with folate and B_{12} was effective in reducing the risk of a hip fracture.

Overall, a large set of clinical trials have shown a negative effect of B_{12} deficiency, while the effect of B_{12} supplementation is dependent on a variety of other confounding factors such as age, sex and genetics. Despite these associations of B_{12} and bone in clinic, the mechanisms through which B_{12} deficiency mediates these skeletal effects in humans are poorly understood.

Animal models of short-term dietary B_{12} deficiency and bone health

To understand how B_{12} deficiency leads to bone abnormalities in humans, studies were carried out on rodents with diets deficient in B_{12}, alone or in combination with other molecules. Herrmann *et al.*[35] developed a rat model of short-term B_{12} and folate deficiency, and showed that it did not affect parameters of bone quality. Similar findings have been reported in a mouse model with short-term deficiency in B_{12} and/or folic acid. Holstein *et al.*[36] using a mouse model of dietary B_{12} deficiency showed that short-term B_{12} deficiency did not alter fracture healing. Consistent with these *in vivo* findings, decreasing B_{12} or folate levels *in vitro* do not affect human osteoblast activity. Together, these studies have shown that short-term dietary B_{12} deficiency does not affect bone quality or fracture healing in mice. These animal or cell culture studies therefore have not been able to illustrate how B_{12} deficiency leads to skeletal abnormalities in humans.

Analysis of the effect of long-term B_{12} deficiency on bone using a mouse genetic model

Gastrointestinal absorption of B_{12} requires an essential protein, *Gif*, produced by the stomach parietal cells. To generate a mouse genetic model of B_{12} deficiency, we recently inactivated *Gif* in the mouse through homologous recombination[6]. Recombination analysis showed that we had successfully inactivated this protein in the mouse. To generate *Gif*–/– animals that are unable to absorb B_{12}, we crossed *Gif*+/– male with *Gif*+/– female generating *Gif*+/+, +/– and –/– offspring. Oral gavage showed that *Gif*–/– offspring were indeed unable to absorb B_{12} from their diet. These *Gif*–/– offspring contained 20-fold less B_{12} in their blood; yet they showed significant amount of B_{12} in their serum. This level of B_{12}, although extremely low, was sufficient to support offspring growth and bone mass up to one year of age; at this point these mutants showed osteoporosis[6]. Serum B_{12} in the first generation of *Gif*–/– offspring was derived from the placental transfer from their *Gif*+/– mothers during gestation. With the reasoning that further depletion of this B_{12} store will lead to more severe and early onset of osteoporosis, we next used F1 *Gif*–/– females as mothers. This genetic cross further lowered serum B_{12} in the F2 *Gif*–/– offspring and led to growth retardation and early onset osteoporosis as early as four weeks of age. These mouse genetic crosses using *Gif*+/– and *Gif*–/– mothers producing first (F1) and second (F2) generation of *Gif*–/– mice indicated that *Gif*–/– (F2) and not *Gif*–/– (F1) animals show consequences of B_{12} deficiency during early postnatal growth, suggesting that maternally derived B_{12} is a powerful determinant of growth and bone mass in the offspring. To further confirm the importance of maternally derived B_{12} in the regulation of bone mass, we gave a single injection of B_{12} to the *Gif*–/– mother during gestation day 14.5. This was sufficient to cure growth retardation and osteoporosis in the *Gif*–/– (F2) mice, confirming the importance of B_{12} derived from the mother in this process. These studies using F1 and F2 *Gif*–/– are consistent with earlier studies that have shown no effect of short-term B_{12} deficiency in animal models, and provided a model in which effect of B_{12} deficiency on skeletal and other abnormalities could be further studied.

B_{12} deficiency specifically affects osteoblast numbers and bone formation

Bone histology and histomorphometry analysis revealed that B_{12} deficiency profoundly affected osteoblast numbers and bone formation, but had no effect on bone resorption parameters[6]. These results suggested that B_{12} deficiency may directly affect osteoblast cells. Depletion of B_{12} in the culture medium for 24 h from 10,000 to 0 ng/ml did not lead to any significant negative effect on

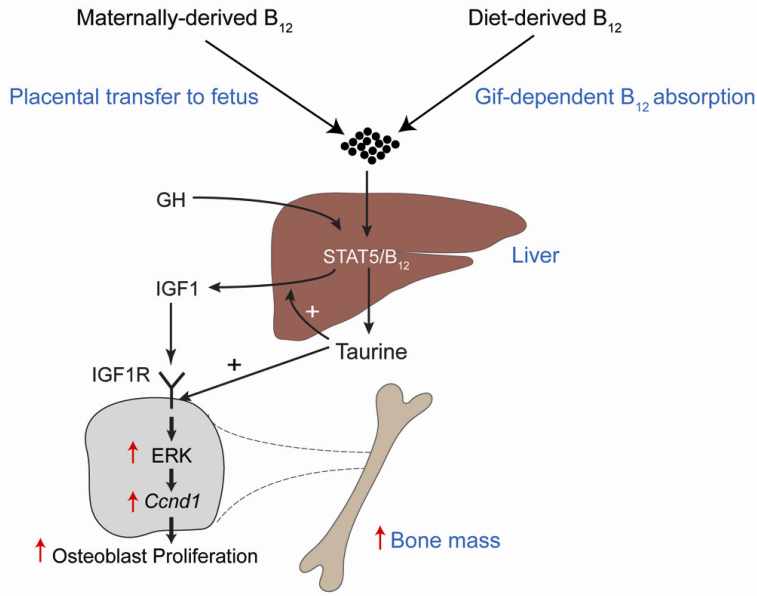

Figure 3. Mechanistic model of B_{12} regulation of bone mass. Vitamin B_{12} is either derived from the mother during early childhood or is absorbed from a diet rich in animal products. B_{12} is then stored in the liver and recycled. In the liver B_{12} affects the synthesis of taurine which positively regulates bone mass through GH–IGF1 axis.

osteoblast proliferation or function. This could easily be anticipated given high stability of B_{12} in animal cells. Moreover, animal cells in their life are never exposed to this severe depletion of B_{12} in the extracellular fluid, including in humans, where serum B_{12} levels below 200 pM are classified into B_{12} deficiency. Given these surprising negative effects of B_{12} deficiency on osteoblasts *in vivo* but not *in vitro*, we examined the organs that might be affected by B_{12} deficiency and could explain low osteoblasts numbers in these mutant mice.

B_{12} deficiency causes growth hormone resistance

Given that B_{12}-deficient animals showed growth retardation during early postnatal life, we examined the levels of growth hormone (GH), a hormone that regulates growth profoundly during peripubertal period. GH regulates various biological processes by either directly acting on the target cell types or by indirectly regulating production of insulin-like growth factors 1 (IGF1) from the liver[37]. For instance, GH regulates bone mass indirectly through production of liver IGF1 that in turn acts on osteoblasts to increase bone mass. Analysis of GH–IGF1 axis in B_{12}-deficient animals revealed that these mutants had two- to three-fold high levels of GH in their serum but very low levels of IGF1, suggesting a state of GH resistance in B_{12}-deficient animals. These results suggest a model in which B_{12} is an essential molecule that regulates liver GH responsiveness to produce IGF1, which in turn acts on the osteoblasts to regulate their proliferation and bone mass. We reasoned that if we normalize the low levels of IGF1 in B_{12} deficient animals, it may rescue growth retardation and low bone mass observed in them. To address this, we next administered twice-daily recombinant IGF1 that has been shown earlier to rescue growth retardation in the state of GH resistance[6]. Surprisingly, however, IGF1 administration in B_{12}-deficient animals led to their death within a few days following the start of the treatment. Further investigation into the cause of death of B_{12}-deficient animals following IGF1 administration led to the discovery that GH resistance was associated with hypoglycaemia in B_{12}-deficient animals and IGF1 injections further decreased their glycaemic state likely leading to the death of these animals. These results together suggest that absence of at least one other molecule in B_{12}-deficient animals besides IGF1 is responsible for their growth retardation and low bone mass.

Taurine deficiency underlies growth hormone resistance and low bone mass in B_{12} deficient animals

Unbiased metabolomics analysis of B_{12}-deficient mice liver led to the identification that these animals have a major reduction in a variety of metabolites[6]. Further downstream analysis of the metabolomics data, which included variable importance in projection analysis, showed that taurine was the most highly dysregulated molecule in B_{12}-deficient livers and that it could separate wild-type (WT) animals from B_{12}-deficient animals with the highest accuracy. Taurine is an atypical amino acid that contains sulphonyl group in place of carboxyl group, and therefore

is not incorporated into proteins. Taurine is synthesized in the animal livers, but can also be obtained in diet. Despite the fact that taurine is present in abundant amounts in the animal body, its function is poorly understood. Further *in vitro* investigations using a liver cell line (HepG2) revealed that GH positively regulated taurine synthesis in hepatocytes and that B_{12} deficiency abrogated GH-dependent taurine synthesis. Further addition of taurine in the culture medium of B_{12}-deficient hepatocytes rescued their GH resistance and restored IGF1 synthesis in these cells. Given these promising *in vitro* results, we tested the model in which taurine was an essential factor downstream of B_{12} that regulated growth and bone mass *in vivo* through the regulation of the GH–IGF1 axis. To test this hypothesis, we gavage-fed B_{12}-deficient animals with taurine daily from day-16 post-birth before the onset of growth retardation. Taurine-fed *Gif*–/– (F2) animals grew as well as WT mice, demonstrating that taurine is an essential factor that can rescue B_{12} deficiency-induced growth retardation and low bone mass. Taurine does so by increasing IGF1 synthesis from the liver and promoting IGF1 action on the osteoblasts. In the osteoblasts taurine promotes IGF1R signalling by increasing the phosphorylation of IGF1R and other downstream signalling proteins. Together, these studies revealed that one mechanism through which B_{12} regulates growth and bone mass is through the regulation of IGF1 and taurine production from the liver downstream of GH (Figure 3)[38].

B_{12}–taurine–bone axis is operational in humans

The demonstration of taurine as a factor downstream of B_{12} that regulates osteoblasts functions in a mouse model of B_{12} deficiency begged the question whether this axis is operational in humans. This was addressed in a small cohort of patients that had B_{12} deficiency either due to maternal insufficiency of B_{12} or due to age-related decline in B_{12} absorption. Correlation analysis between serum levels of B_{12}, taurine and osteocalcin showed that like the B_{12}-deficient mouse model, patients had a significant positive correlation between these parameters. These clinical studies provided evidence that B_{12}–taurine–bone axis is operational in humans as it is in mice[6].

Summary

The study on the regulation of growth and bone disorders through long-term B_{12} deficiency has led to the discovery of an important role played by the liver in this process. These findings have led to the identification of taurine as an important amino acid produced by liver, whose function in the whole-body homeostasis is only now beginning to be understood. Multiple evidences point towards the fact that the manipulation of this gut–liver bone axis has the potential to increase bone mass in low bone mass disorders. First, B_{12} and taurine specifically regulate bone formation and therefore have the potential to increase bone formation in diseases that specifically affect this process. Second, both these products are naturally derived and therefore use of these molecules will likely pose no safety issues. For instance, taurine is produced in the body and B_{12} is stored in the body in high amounts in the liver. However, as in other discoveries of novel pathways, we have a long way to go before the true potential of B_{12}–taurine axis in the treatment of bone and other metabolic disorders will be realized.

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