

Metformin revisited: Does this regulator of AMP-activated protein kinase secondarily affect bone metabolism and prevent diabetic osteopathy?

Antonio Desmond McCarthy, Ana María Cortizo, Claudia Sedlinsky

Antonio Desmond McCarthy, Ana María Cortizo, Claudia Sedlinsky, Laboratorio de Investigaciones en Osteopatías y Metabolismo Mineral, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata 1900, Argentina

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Correspondence to: Antonio Desmond McCarthy, Professor, Laboratorio de Investigaciones en Osteopatías y Metabolismo Mineral, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Calle 50 y 115, La Plata 1900, Argentina. mccarthy@biol.unlp.edu.ar
Telephone: +54-221-4512426
Fax: +54-221-4512426

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Abstract

Patients with long-term type 1 and type 2 diabetes

mellitus (DM) can develop skeletal complications or "diabetic osteopathy". These include osteopenia, osteoporosis and an increased incidence of low-stress fractures. In this context, it is important to evaluate whether current anti-diabetic treatments can secondarily affect bone metabolism. Adenosine monophosphate-activated protein kinase (AMPK) modulates multiple metabolic pathways and acts as a sensor of the cellular energy status; recent evidence suggests a critical role for AMPK in bone homeostasis. In addition, AMPK activation is believed to mediate most clinical effects of the insulin-sensitizer metformin. Over the past decade, several research groups have investigated the effects of metformin on bone, providing a considerable body of pre-clinical (*in vitro*, *ex vivo* and *in vivo*) as well as clinical evidence for an anabolic action of metformin on bone. However, two caveats should be kept in mind when considering metformin treatment for a patient with type 2 DM at risk for diabetic osteopathy. In the first place, metformin should probably not be considered an anti-osteoporotic drug; it is an insulin sensitizer with proven macrovascular benefits that can secondarily improve bone metabolism in the context of DM. Secondly, we are still awaiting the results of randomized placebo-controlled studies in humans that evaluate the effects of metformin on bone metabolism as a primary endpoint.

Key words: Diabetes mellitus; Osteoporosis; Bone fractures; Metformin; AMP-activated kinase

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Core tip: Patients with long-term type 1 and type 2 diabetes mellitus (DM) can develop skeletal complications. These include osteopenia, osteoporosis and increased incidence of low-stress fractures. In this context, it is important to evaluate whether current anti-diabetic treatments can secondarily affect bone metabolism. Over the past decade, several research

groups have investigated the effects of metformin on bone, providing a considerable body of pre-clinical (*in vitro*, *ex vivo* and *in vivo*) as well as clinical evidence for an anabolic action of metformin on bone. This could be particularly relevant when considering treatment options for DM in the context of diabetic osteopathy.

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INTRODUCTION

Diabetes mellitus (DM) is a highly prevalent global disease associated with long-term microvascular and macrovascular complications. Over the past 30 years, an increasing body of experimental and clinical evidence has reported the association of type 1 and type 2 DM with osteopenia, osteoporosis and an increased incidence of low-stress fractures, in what has been called diabetic osteopathy^[1]. Many adult patients with type 1 DM show mild osteopenia, with a decrease in bone mineral density (BMD) of around 10%^[2] that would be expected to double the risk of non-vertebral fragility fractures^[3]. However, the incidence of low-stress fractures in type 1 DM is 7-12 times that of age-matched non-diabetic individuals^[4,5]. On the other hand, patients with type 2 DM tend to have normal or even moderately elevated BMD. Although this would be expected to reduce their incidence of osteoporotic fractures, they actually show a 2-fold increase in hip, extremity and vertebral fractures^[3-7]. Taken together, these clinical observations are considered to be evidence for a significant decrease in bone quality of patients with both types of DM^[8] that would explain their increase in low-stress fractures.

Several mechanisms have been proposed to explain diabetic osteopathy, such as disturbed glucose metabolism, tissue (bone) and systemic low-grade inflammation, changes in the secretory pattern of growth factors and/or cytokines, increased oxidative stress and excess accumulation of advanced glycation end products (AGEs). In particular, excess accumulation of AGEs in bone extracellular matrix (ECM) occurs as a function of aging and duration of Diabetes, and has been found to impair the mechanical properties of bone^[9]. Poorly compensated DM elevates circulating reactive oxygen species (ROS), glucose and/or carbonyl stress, which can induce excess AGEs formation on bone ECM, reducing bone strength and post-yield properties. Additionally, collagen-AGEs interact with the receptor for AGEs (RAGE) expressed by osteoblasts and osteoclasts, inhibiting their functionality and decreasing bone turnover. This induces an even greater accumulation of AGEs in bone that contributes to

diabetic osteopathy, and can increase fracture risk^[1].

Treatment of patients with DM can include either an absolute requirement for exogenous insulin (type 1 DM), or relative requirement of glucose-lowering medication such as insulin and/or oral drugs (type 2 DM). Oral glucose-lowering agents fall into different classes that include (but are not limited to) insulin secretagogues, insulin sensitizers, incretin-based treatments and inhibitors of renal proximal tubule glucose reabsorption. Each class operates through distinct biological pathways and has certain advantages as well as disadvantages. Recently, several commonly prescribed oral medications for type 2 DM have been found to secondarily affect bone metabolism, in some cases modifying the incidence of fragility fractures^[10]. Depending on their specific skeletal effects these drugs could either help to prevent diabetic osteopathy, or contribute to worsen this complication of DM.

Members of the thiazolidinediones (TZD) family of insulin-sensitizers such as rosiglitazone have been shown to be detrimental for bone health. In rodents, TZD increase the adipocytic commitment of mesenchymal stem cells (MSC) while decreasing their osteogenic potential, *via* a decrease in the Runx2/PPAR γ ratio. This increases bone marrow adiposity and promotes bone loss^[11]. In the ADOPT clinical trial^[12], results showed a higher risk of fracture in diabetic women, but not men, on rosiglitazone monotherapy.

Post-prandial incretin secretion is believed to play a physiological role linking nutrient ingestion to suppression of bone resorption and stimulation of bone formation. Thus, incretin-based treatments would be expected to show anabolic effects on bone, as has been suggested in a recent meta-analysis^[13]. However, this may not be so in certain cases such as the DPP4 inhibitor saxagliptin, which has been found to impair MSC osteogenic potential and bone micro-architecture in rodent models^[14].

Inhibitors of the sodium glucose cotransporter 2 such as dapagliflozin and canagliflozin, that decrease plasma glucose and body weight by impairing proximal tubule glucose reabsorption, have recently been associated with alterations in mineral metabolism and with an increase in bone fractures. This undesirable effect is probably due to the fact that these drugs can induce hyperphosphataemia and an increase in fibroblast growth factor-23 and para-thyroid hormone levels^[15].

Metformin is an insulin-sensitizing biguanide that was developed several decades ago; however, it is still the most widely used oral anti-diabetic medication, particularly since the United Kingdom Prospective Diabetes Study demonstrated its efficacy for reducing macrovascular complications in obese type 2 DM patients^[16]. Although the exact mechanism of action for metformin is still incompletely understood, in various tissues and organs it improves glucose metabolism *via* activation of the ubiquitously expressed AMP-activated protein kinase (AMPK)^[17,18]. AMPK subunit expression and activation is tissue-specific, with the α 1 subunit

accounting for most of bone AMPK^[19]. Over the last 10 years, pre-clinical and clinical evidence has accumulated pointing to an anabolic effect of metformin on bone, in part due to AMPK activation.

AMPK-A KEY ENERGY SENSOR

AMPK is a Ser/Thr protein kinase that modulates multiple metabolic pathways and acts as a sensor of the cellular energy status^[20]. AMPK is a heterotrimer of three subunits. The α subunit holds the catalytic domain with a Ser/Thr kinase domain (KD). It contains a Thr172 residue, critical for the kinase activity. AMPK- α also contains a regulatory domain, which interacts with the KD of the unphosphorylated inactive form. The β subunit contains a carbohydrate-binding module and acts as a scaffold for the assembly of α and γ subunits. It also defines the subcellular localization of AMPK as well as its substrate specificity. The γ subunit contains four cystathionine- β -synthase (CBS) domains, which bind adenine nucleotides. Expression of all three subunits is required for AMPK activity. The mechanism of activation first requires the reversible phosphorylation of the α subunit; then after AMP binding to the CBS domain of γ subunit, an allosteric stimulatory effect occurs. Several upstream kinases (AMPKK) are involved in the phosphorylation of AMPK, such as liver kinase B1 (LKB1) and Ca²⁺/calmodulin-dependent protein kinase beta (CaMKK β). Activation of AMPK can occur by two mechanisms: One is mediated by an increase of AMP/ATP ratio (for instance during exercise), so this mechanism coordinates anabolic and catabolic pathways to equilibrate nutrient supply with energy expenditure^[21]. Several compounds can activate AMPK by this mechanism: 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), H₂O₂, MAPK inhibitors, TZD, leptin, adiponectin and α -lipoic acid^[22]. In the second mechanism, AMPK activation can be independent of AMP/ATP ratio and involves alternative AMPK regulation. Agents such as peroxynitrite, metformin, estradiol, low glucose levels and several membrane receptor agonists can induce AMPK activation by this way.

It has recently been demonstrated that AMPK can participate in the control of whole-body energy homeostasis by integrating signals from diverse cellular environments^[23]. AMPK participates in several physiological events, such as survival, growth and development. This kinase could also be implicated in pathological conditions such as type 2 DM, insulin resistance, cardiovascular disease and cancer. For instance, AMPK can control epigenetic processes in certain cells to avoid reproductive defects in their subsequent generations^[24]; or it can suppress proinflammatory-signalling pathways in adipocytes^[25]. It has recently been reported that in patients with insulin resistance, AMPK is depressed in adipose tissue; an effect that is associated with oxidative stress, increased expression of inflammatory cytokines and decreased expression of genes regulating oxidative

phosphorylation. On the contrary, AMPK activation increases mitochondrial biogenesis^[26]. AMPK can also inhibit the proliferation of muscle stem cells, and induce the differentiation of endothelial cell progenitors^[21].

ROLE OF AMPK IN CELL METABOLISM

AMPK functions as an intracellular sensor regulating energy balance in different cell types, and thus can regulate diverse metabolic pathways (Table 1).

In the liver, AMPK acts as a "metabolic master switch"^[27]; its activation inhibits energy-consuming pathways and stimulates ATP-producing catabolic pathways. For instance, after AMPK activation in a fasting state, fatty acid synthesis is inhibited while mitochondrial oxidative phosphorylation is stimulated. These effects occur by a reduction in malonyl-CoA content that is mediated by inhibition of Acetyl-CoA carboxylase. In addition, malonyl CoA decarboxylase is activated, thus further increasing fatty acid oxidation. Activation of AMPK also suppresses glucose production (gluconeogenesis), as has been demonstrated in metformin-treated primary hepatocyte cultures^[17]. This has been confirmed in mice with a liver-selective deletion of the *AMPK α 2* gene, which exhibit hyperglycaemia and glucose intolerance in the fasting state^[27].

In skeletal muscle, AMPK regulates energy expenditure during exercise in order to optimize and enhance energy production. It participates in the transition from more glycolytic fibres to more oxidative fibres, following exercise training^[28]. A role for AMPK in the myocytic uptake and oxidation of fatty acids has also been postulated. In addition, during exercise an increase in ATP turnover is accompanied by enhanced glucose uptake, in turn associated with an increase in myocyte plasma membrane GLUT4 expression^[21]. Thus, use of metformin in patients with type 2 DM will increase their AMPK-induced glucose uptake and disposal. AMPK activation has also been postulated to induce skeletal muscle regeneration, by regulating its post-injury inflammatory response^[29]. Postnatal skeletal muscle regeneration involves stem cell reprogramming that induces their proliferation, differentiation and/or self-renovation, and activation of the AMPK pathway is believed to regulate these processes^[21]. Due to the regulatory effects of AMPK on integrated metabolism, activation of AMPK is considered a therapeutic target for hyperglycaemic states. For instance metformin, an anti-diabetic drug that is widely used for treatment of patients with type 2 DM, suppresses hepatic glucose production and decreases plasma glucose levels *via* activation of AMPK pathways.

The precise effects of AMPK on bone metabolism are incompletely known; however, recent evidence supports an active role for this kinase in bone physiology^[30]. Several reports have demonstrated that AMPK modulates bone cell differentiation and function. In AMPK α -deleted animals, a reduction was found in

Table 1 Role of AMP-activated protein kinase activation on cell metabolism in different organs

Organ	Effect	Mechanism of action	Ref.
Liver	Inhibition of anabolic pathways	Inhibition of fatty acid synthesis Inhibition of gluconeogenesis	[27]
Skeletal muscle	Stimulation of ATP synthesis	Stimulation of Mitochondrial oxidative phosphorylation	[27]
	Regulation of energy expenditure during exercise	Favours the transition from glycolytic to oxidative skeletal muscle fibers	[28]
		Regulation of myocytic uptake and oxidation of fatty acids	[21]
		Enhanced glucose uptake <i>via</i> an increase in GLUT4 expression	[21]
Bone	Increase in skeletal muscle regeneration	Regulation of post-injury inflammatory response	[29]
	Increase in osteoblastogenesis	Stem cell reprogramming: Induction of proliferation, differentiation and self-renewal	[21]
		Increases MSC differentiation towards the osteoblastic lineage favouring Runx2 expression	[30,37]
		Decreases PPAR γ expression diminishing MSC differentiation towards the adipocytic phenotype	[36,37]
	Decrease in osteoclastogenesis	Negative regulation of RANKL expression by osteoblasts	[35]

MSC: Mesenchymal stem cells; ATP: Adenosine triphosphate; GLUT4: Glucose transporter type 4; RANKL: Receptor activator for nuclear factor κ B ligand.

trabecular bone mass^[31]. Single α 1 or α 2 knockout (KO) mice are viable, but the double KO is embryonically lethal^[27]. In addition, histomorphometric analysis revealed that AMPK α 1 KO mice show an elevated rate of bone remodelling *in vivo*, associated with increased osteoclastogenesis *in vitro*.

In vitro experiments have demonstrated that AMPK activation enhances osteogenesis^[30,32] while compound C (an AMPK inhibitor) reduces osteoblastic mineralization^[30]. In other experiments AICAR (an activator of AMPK) was found to stimulate alkaline phosphatase activity (ALP) and mineral nodule formation by rat calvaria-derived cells, while compound C suppressed these effects^[33]. A decrease in AMPK activity has been reported during osteoblastic differentiation; this could be associated with the high-energy requirements of maturing osteoprogenitor cells^[34]. Although studies of AMPK action on bone resorption have led to conflicting results, it appears that AMPK is a negative regulator for RANKL and can thus decrease osteoclast-mediated bone resorption^[35]. The AMPK pathway may also be involved in regulating the fate of bone marrow stromal cells (MSC) toward the osteoblastic or adipocytic lineage by reciprocally regulating the expression of Runx2 and PPAR γ ^[30]. AMPK has been shown on one hand to induce phosphorylation of β -catenin, suppress PPAR γ expression and thus reduce adipogenesis^[36]; while on the other hand it increases Runx2 expression and thus osteoblastic differentiation of MSC^[37]. This evidence suggests a critical role for AMPK in bone homeostasis.

also been postulated^[38].

Metformin can be incorporated into cells by a facilitated mechanism that is mediated by different isoforms of the organic cation transporter. Metformin induces mild and specific inhibition of the mitochondrial respiratory chain complex in hepatocytes and other tissues^[39]; it can also inhibit the mitochondrial production of ROS. Inhibition of mitochondrial activity induces a decrease in the cell energy status, which in turn triggers depletion of ATP and a diminished ATP/AMP ratio. This effect then induces phosphorylation and activation of AMPK *via* LKB1. Metformin also induces an acute inhibition of gluconeogenesis: this can be explained by the decrease in ATP/AMP ratio, which inhibits key enzymes of the gluconeogenic pathway such as fructose-1,6-bisphosphatase. New evidence suggests that this metformin-induced inhibition of glucose production could also be mediated by a down-regulation of gluconeogenic genes *via* a transcription-independent process. It has been suggested that reduction in energy status, but not AMPK activation, is critical for metformin inhibition of hepatic glucose production^[38].

In addition to its effects in the liver, metformin can also affect other organs *via* multiple molecular mechanisms. One important action of metformin is its reduction of endothelial activation and of atherogenesis^[16]. Metformin decreases intracellular ROS production in endothelial cells by inhibiting both NADPH oxidation and the respiratory chain complex^[40], and this effect appears to be independent of AMPK activation.

MOLECULAR MECHANISMS OF METFORMIN ACTION

Metformin has been widely used in the United States since 1995 as an oral anti-diabetic treatment for type 2 DM^[22]. Even though its precise mechanism of action is not completely known, metformin is known to activate AMPK^[17]; however, AMPK-independent pathways have

METFORMIN MEETS BONE

Over the past decade, several research groups have investigated the effects of metformin on bone. The results of these studies are discussed in detail below. They have provided a considerable body of pre-clinical (*in vitro*, *ex vivo* and *in vivo*) as well as clinical evidence for an anabolic action of metformin on bone, which could be particularly relevant when considering treatment

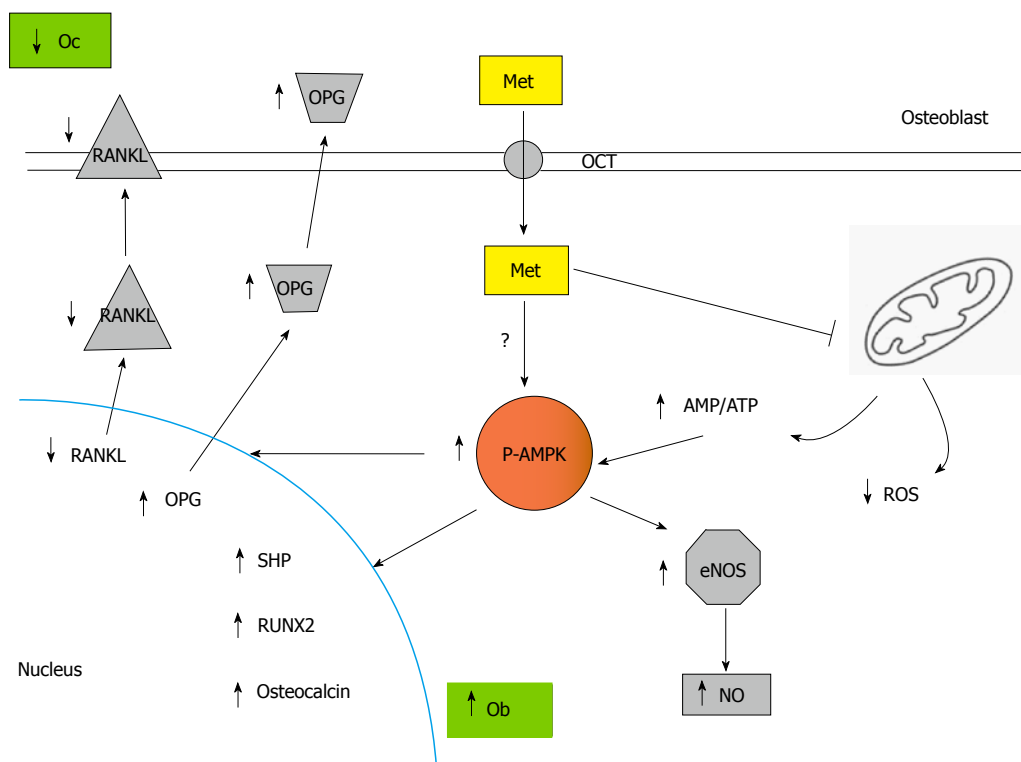


Figure 1 Metformin actions via osteoblasts are pro-osteogenic and anti-resorptive. Metformin is incorporated into osteoblasts, where it inhibits intracellular ROS production and induces AMPK phosphorylation/activation. This increases eNOS activity and NO production, promoting osteoblast proliferation. In addition, activated AMPK up regulates osteoblastic differentiation and mineralization via expression of Runx2 and SHP, while decreasing osteoclastic recruitment and bone-resorbing activity through a reduction in osteoblastic RANKL/OPG ratio. ROS: Reactive oxygen species; AMPK: AMP-activated protein kinase; OPG: Osteoprotegerin; RANKL: Reduced receptor activator of nuclear factor- κ B ligand; SHP: Small heterodimer partner; RUNX2: Runt-related transcription factor 2; AMP: Adenosine monophosphate; ATP: Adenosine triphosphate; Met: Metformin.

options for DM in the context of diabetic osteopathy.

In vitro effects of metformin on bone cells

Metformin has been found to modulate the physiology of osteoblasts (Figure 1) and osteoclasts, as well as influencing the phenotypic progression of bone MSC.

Cortizo *et al*^[41] were the first to describe an *in vitro* effect of metformin on bone-derived cells, showing that it dose-dependently increased osteoblastic proliferation, differentiation and mineralization. This effect was mediated by activation of extracellular-regulated kinases and by induction of NO synthases. Several researchers have corroborated these results^[19,42-44], additionally showing that the osteogenic *in vitro* action of metformin on osteoblasts is dependent on activation of the AMPK signalling pathway and subsequent bone morphogenetic protein-2 production. In an interesting mechanistic study, Jang *et al*^[45] found that metformin increased the osteoblastic transcription of small heterodimer partner (SHP) and osteocalcin genes, an effect that was inhibited either by a dominant negative form of AMPK or by compound C. They also found that metformin-induced SHP gene expression was mediated by upstream stimulatory factor-1 (USF-1), that AMPK activation increased the expression of Runx2 and that SHP interacts physically and forms a complex with Runx2 on the osteocalcin gene promoter in osteoblastic cells. Thus, metformin appears to enhance osteoblast

differentiation through the transactivation of Runx2 via the AMPK/USF-1/SHP regulatory cascade^[45]. In another study, Mai *et al*^[46] found that metformin dose-dependently stimulated osteoprotegerin (OPG) and reduced receptor activator of nuclear factor- κ B ligand (RANKL) mRNA and protein expression by cultured osteoblastic cells, a potentially anti-osteoclastogenic effect that they were able to block by inhibition of AMPK^[46].

Since one of the proposed mechanisms for diabetic osteopathy is hyperglycaemia-mediated accumulation of AGEs on bone collagen, and AGEs can decrease osteoblastic maturation and survival via binding to their receptor RAGE, Schurman *et al*^[47] investigated whether this process could be modulated *in vitro* by metformin. They found that metformin was able to prevent the increase in apoptosis, caspase 3 activity, inhibition of ALP and alterations in intracellular oxidative stress induced by AGEs in osteoblastic cells, via a metformin-dependent down regulation in the osteoblastic expression of RAGE^[47]. In another study, Zhen *et al*^[33] evaluated whether metformin could prevent the anti-proliferative effect of high-glucose exposure on primary osteoblasts in culture. They found that incubation with metformin decreased the high-glucose-induced intracellular ROS production and apoptosis, and that it additionally induced an osteogenic effect on osteoblasts that was mediated by an increase in Runx2 and IGF-1

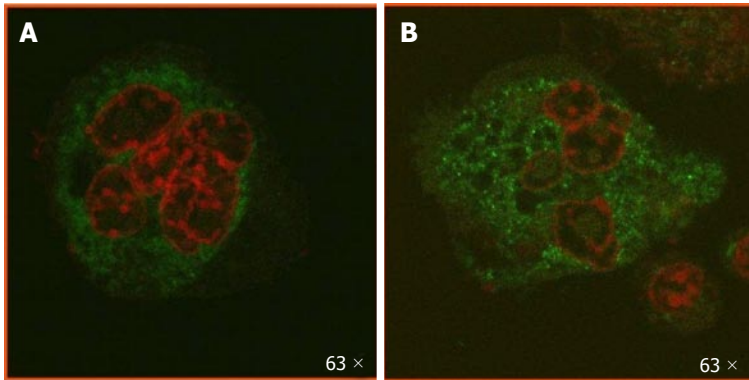


Figure 2 Metformin induces an increase and redistribution of activated AMP-activated protein kinase in multinucleated osteoclasts. UMR106 osteoblasts and Raw 264.7 macrophages were co-cultured for 7 d, in the absence (A) or presence (B) of 500 mol/L metformin. Cells were fixed, permeabilized and incubated with an anti-phosphorylated AMPK antibody, followed by a FITC-conjugated secondary antibody (green). Nuclei were counterstained with propidium iodide (red). Cells were visualized with a Leica TSC SP5 AOBS confocal microscope. Metformin induced an increase in activated AMPK with a punctillate and predominantly cytoplasmic distribution. AMPK: AMP-activated protein kinase.

gene expression^[33]. These results have been recently confirmed by other investigators^[48].

Three different studies have investigated the *in vitro* effects of metformin on stromal cells isolated from bone marrow (MSC). In the first report, Gao *et al.*^[49] found that metformin increased Runx2 and decreased PPAR γ expression, and consequently stimulated ALP and mineralization while inhibiting the intracellular accumulation of lipid droplets. These results suggest that metformin could influence the reciprocal relationship between osteoblastic and adipogenic differentiation of MSC, tipping the balance towards osteogenesis. Molinuevo *et al.*^[37] further demonstrated that metformin could induce an *in vitro* dose-dependent increase in MSC osteogenic potential (ALP, type 1 collagen secretion and mineralization). They also found that metformin dose-dependently prevents rosiglitazone-induced intracellular lipid accumulation by MSC^[37]. In another study, Sedlinsky *et al.*^[50] demonstrated that the *in vitro* osteogenic effect of metformin on MSC is AMPK-dependent, and that it can be completely blocked by the AMPK inhibitor compound C.

Metformin has also been found to modulate *in vitro* osteoclast recruitment, differentiation and bone-resorbing activity in some^[35,46,51] but not all^[52] published reports. OPG and RANKL are predominantly secreted by osteoblasts and play critical roles in osteoclast physiology. As stated above, *in vitro* experiments have shown that metformin increases OPG and reduces RANKL mRNA and protein expression by osteoblasts, which is potentially anti-osteoclastogenic. Additionally, when a macrophage cell line was incubated with the supernatant of osteoblasts treated with metformin, this reduced the formation of tartrate resistant acid phosphatase (TRAP)-positive multi-nucleated osteoclasts^[46]. In another interesting *in vitro* study, AMPK was found to be expressed by bone marrow pre-osteoclasts and as such is a regulatory target for osteoclast differentiation and resorptive activity. Pharmacological inhibition of pre-osteoclastic AMPK with compound

C increased the RANKL-induced formation of TRAP-positive multinucleated cells and their resorptive activity on dentine discs, *via* downstream activation of p38, JNK, NF- κ B, Akt, CREB, c-Fos, and NFATc1. On the contrary, metformin dose-dependently suppressed formation of TRAP-positive multinucleated cells and dentine resorption^[35]. In unpublished results using indirect immunofluorescence, we have found a metformin-induced increase and sub-cellular redistribution in phosphorylated (activated) AMPK of multinucleated osteoclasts obtained from osteoblast-macrophage co-cultures (Figure 2), which could be mediating the effects of metformin in this cell type.

All in all, these *in vitro* results point to a global bone-anabolic effect of metformin: Tipping the phenotypic balance of bone MSC towards osteoblastogenesis, increasing the bone-forming capacity of osteoblasts, and decreasing the recruitment and bone-resorbing activity of osteoclasts (Figure 3). These findings are further supported by *in vivo* and *ex vivo* (pre-clinical) as well as clinical evidence, pointing to an osteogenic action of metformin in the context of DM.

***In vivo* and *ex vivo* effects of metformin on bone metabolism: Animal models**

Most studies using animal models (but not all) have shown beneficial actions of metformin on bone metabolism and on bone lesion repair (Figure 4).

Molinuevo *et al.*^[37] demonstrated an *ex vivo* osteogenic effect of metformin: *i.e.*, that bone MSC obtained from rats after a 2-wk treatment with oral metformin, exhibit increased osteogenic potential (Runx2 expression, ALP activity, type 1 collagen production, osteocalcin synthesis, and mineral nodule deposition) vs MSC obtained from non-treated animals. In addition, these metformin-induced effects were found to be secondary to AMPK activation. In this study, metformin treatment also stimulated the repair of a minimal parietal lesion *in vivo*, both in diabetic and non-diabetic rats.

As stated above, rosiglitazone is a TZD that induces

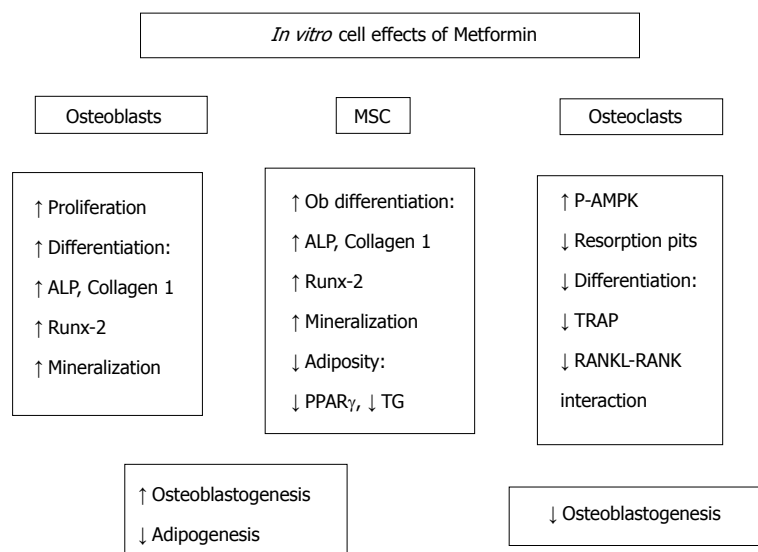


Figure 3 Effects of metformin on bone-derived cells. The results of several *in vitro* studies show that metformin modulates the phenotypic balance of bone marrow stromal cells (MSC) away from adipogenesis and towards osteoblastogenesis. In addition, metformin increases *in vitro* the bone-forming capacity of osteoblasts, while decreasing the recruitment and bone-resorbing activity of osteoclasts. ALP: Alkaline phosphatase; TG: Triglycerides; TRAP: Tartrate-resistant acid phosphatase; RANK: Receptor activator for nuclear factor κ B; RANKL: RANK ligand.

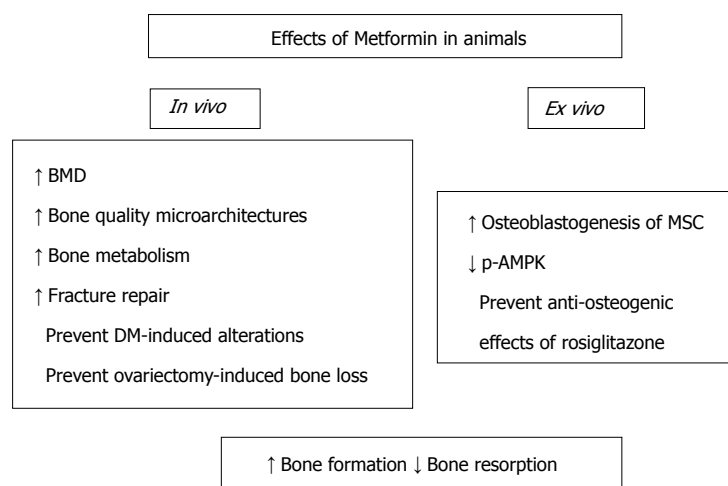


Figure 4 Actions of metformin on bone metabolism - animal studies. Orally administered metformin promotes the osteogenic potential of bone MSC, increases the quality of bone tissue (improving its micro-architecture and mineral density) and facilitates the repair of bone lesions. In addition, metformin may prevent experimental diabetic osteopathy as well as ovariectomy-induced bone loss. MSC: Marrow stromal cells; BMD: Bone mineral density; p-AMPK: Phosphorylated AMP-activated kinase.

deleterious effects on osteoblast differentiation^[53] and on osteocyte survival^[54], diverting MSC differentiation toward the adipocyte lineage. In view of the opposite effect that has been demonstrated for metformin on these cell types, Sedlinsky *et al.*^[50] investigated the effect of a 2-wk metformin/rosiglitazone combined oral treatment of rats on long-bone metaphyseal microarchitecture, minimal parietal lesion repair and MSC osteogenic potential. Compared to untreated controls, rosiglitazone monotherapy decreased femoral metaphysis trabecular area, osteoblastic and osteocytic density, and TRAP activity associated with epiphyseal growth plates. In addition, it greatly diminished bone repair. It also decreased the *ex vivo* osteogenic potential of MSC, inducing an increase in PPAR γ and a

decrease in Runx2 expression, as well as a decrease in phosphorylated (active) AMPK. Metformin/rosiglitazone co-treatment prevented all the *in vivo* (bone repair and diaphyseal microarchitecture) and *ex vivo* anti-osteogenic effects of rosiglitazone monotherapy, with a reversion back to control levels of PPAR γ , Runx2 and AMPK phosphorylation in MSC^[50].

In another study, the skeletal (femoral) effects of rosiglitazone were compared to those of metformin in insulin-resistant female C57BL6J *ob/ob* mice. The metformin-treated group showed higher BMD, higher trabecular bone volume/total bone volume, higher osteoid width and mineral apposition, lower trabecular spacing and lower bone marrow adiposity, when compared with the rosiglitazone-treated group^[55].

Gao *et al.*^[56] studied the *in vivo* effect of oral metformin on bone mass in ovariectomized rats. They found that metformin dose-dependently reverted ovariectomy-induced bone loss, showing an improvement in BMD measured by DEXA, and in bone microarchitecture measured both by micro CT and by bone histology. By real-time PCR of MSC, they found a metformin-dependent increase in Runx2 and Lrp5 (co-receptor for Wnt) expression, both of which are involved in osteoblastic proliferation and differentiation^[56].

In another report, Jeyabalan *et al.*^[57] studied either ovariectomized C57BL/6 mice or young Wistar rats to evaluate the effect of oral metformin on bone metabolism and fracture repair, respectively. In both models, metformin did not modify bone microarchitecture or cellular activity *in vivo* as evaluated by micro-CT and bone histomorphometry. In addition, metformin had no significant effect on the repair of a midshaft femoral fracture in Wistar rats^[57].

Mai *et al.*^[46] further investigated the effects on bone of an oral metformin treatment in ovariectomized (OVX) adult rats. They found that metformin treatment of OVX animals significantly increased total body BMD, enhanced bone mineral content and decreased trabecular separation; supporting the concept that metformin can prevent OVX-induced bone loss. The authors also found that metformin reverted the OVX-associated increase in TRAP-positive osteoclasts of proximal tibiae resorption pits. Metformin treatment also increased serum OPG, and decreased RANKL expression by MSC, in OVX rats. Further *in vitro* experiments showed that these effects were regulated by AMPK and by its upstream activator CaMKK.

In a rat model of partially insulin-deficient nicotinamide/streptozotocin-induced DM, tibia histomorphometry showed a diabetes-induced decrease in trabecular bone volume, osteocyte density, growth plate height and osteoclast (TRAP positive) activity in the primary spongiosa, as well as an increase in bone marrow adiposity. MSC from diabetic animals showed a decrease in their osteoblastic potential, an increase in adipocytic commitment, a reduction in their Runx2/PPAR γ ratio and an increased expression of the AGEs receptor RAGE. A 2-wk oral treatment with metformin prevented all these Diabetes-induced alterations in bone micro-architecture and MSC osteogenic potential, and also induced a down-regulation of RAGE expression by MSC^[58].

Clinical evidences of metformin effects on bone

There are few published clinical studies reporting the skeletal effects of metformin. In addition, randomized placebo-controlled studies in humans that evaluate the effects of metformin on bone metabolism as a primary end point are so far unavailable. The results of published clinical reports are summarized in Table 2.

Several epidemiological studies have reported the effects of diabetes and antidiabetic agents on bone fracture risk. In 2005, Vestergaard *et al.*^[59] published

a Danish population-based study evaluating risk of fractures and its relationship with T1DM and T2DM and anti-diabetic agents. They found that both T1DM and T2DM patients had a significant increase in bone fracture risk, and that the use of metformin was associated with a significantly decreased risk for fracture at any site.

Melton *et al.*^[6] conducted another population-based study in Rochester, United States, to evaluate fracture risk factors in T2DM patients. They found that patients had an increased risk of hip fracture after 10 years of DM, and that use of biguanides such as metformin was protective even after adjusting for other risk factors (HR, 0.7; 95%CI: 0.6-0.96).

Monami *et al.*^[60] conducted a case-control study, nested within a retrospective cohort, comparing 83 case subjects with a history of bone fractures and 249 control subjects, in all cases exposed to insulin, insulin secretagogues or metformin treatment for the past 10 years, in order to assess the risk for bone fractures associated with exposure to insulin or different oral hypoglycaemic agents. This study was unable to demonstrate a reduction in bone fractures associated with metformin treatment, but showed an increased rate of fractures in patients on insulin treatment, probably related to worse diabetes control or to hypoglycaemic episodes. Nevertheless, the authors acknowledged that the lack of a statistically significant fracture reduction associated with metformin treatment was probably related to an insufficient sample size.

The ADOPT study (A Diabetes Outcome Progression Trial), that compared the glycaemic effects of rosiglitazone, metformin and glyburide, showed that among the adverse effects of rosiglitazone was an increased risk of fracture in women. At the same time they showed that metformin had a lower risk of fracture, both in women and men, for every skeletal site assessed^[12]. In an add-on report to the ADOPT study, C-telopeptide levels (CTX, a bone resorption marker) were found to be reduced by metformin treatment and increased in rosiglitazone-treated patients, suggesting that changes in bone resorption may be partly responsible for the differences in fracture risk observed for both treatments^[61].

A randomized, parallel group, double-blind, multicentre study comparing the efficacy and safety of rosiglitazone/metformin co-treatment (RSG/MET) vs metformin monotherapy (MET) was conducted in order to assess glycaemic control and BMD after 80 wk of treatment in drug-naïve T2DM patients. Although the RSG/MET combination was superior to MET in achieving significant reductions in glycated haemoglobin and fasting plasma glucose, RSG/MET was associated with a significantly lower BMD in comparison with MET at week 80 in the hip and lumbar spine^[62].

In another study, Hegazy^[63] evaluated the possible anti-osteoporotic effect of metformin vs sitagliptin in 40 post-menopausal diabetic women. They were randomly divided into two groups, one receiving 500 mg

Table 2 Clinical evidences of metformin effects on bone

Study design	Study population	n	Outcome	Ref.
Case control study	All subjects with bone fracture in Denmark (year 2000), vs 3-fold controls	124655 fracture patients 373962 control	Fracture risk = 0.81 (95%CI: 0.70-0.93) ¹ (for metformin)	[59]
Cohort Study	Rochester residents first meeting Diabetes glycaemic criteria (1970-1994)	1964 diabetic patients	Fracture risk = 0.7 (95%CI: 0.6-0.96) ² (for metformin)	[6]
Case control study	A study nested within a cohort of 1945 diabetic Tuscany outpatients (1998-2004)	83 fracture patients 249 control	Fracture risk = 0.60 (95%CI: 0.34-1.08) ³ (for metformin)	[60]
Double-blind, randomized, controlled clinical trial	Recently diagnosed, drug-naïve patients with type 2 diabetes, treated for a median of 4 yr with rosiglitazone, metformin, or glyburide	Rosiglitazone: n = 1456; Metformin: n = 1454; Glyburide: n = 1441	N ^o Fractures (%): Rosiglitazone 60 (9.30) Metformin 30 (5.08) ⁴ Glyburide 21 (3.47) ⁴	[12]
Double-blind, randomized, controlled clinical trial	Recently diagnosed, drug-naïve patients with type 2 diabetes, treated for a median of 4 yr with RSG, MET, or GLY	Paired baseline and 12-mo stored serum samples from 1605 patients	In women, CTX increased by 6.1% with RSG, decreased by 1.3% with MET (P = 0.03) In men, CTX was unchanged on RSG (-1.0%) and fell with MET -12.7% (P = 0.001)	[61]
Randomized, parallel group, double-blind, multicentre study	Drug naïve, male and female patients who had an established clinical diagnosis of type 2 diabetes mellitus	688 patients equally randomized to RSG/MET or MET	BMD at week 80: Lumbar = (-2.2) (95%CI: -3.5, -0.9) Total hip = (-1.5) (95%CI: -2.3, -0.7) ⁵	[62]
Prospective randomized study with active comparator study	Forty postmenopausal diabetic women recruited from Tanta University Hospitals	20 patients on metformin and 20 on sitagliptin, for 12 wk	BMD was unchanged in both groups at week 12 Bone turnover markers remained unchanged from baseline in MET	[63]
Prospective randomized double-blind, double-dummy with active comparator	Men with uncomplicated type 2 diabetes mellitus, aged 45-65 yr	71 men were randomized to PIO once daily or MET twice daily	Sclerostin levels at week 24 increased by 11% in PIO-treated patients and decreased by 1.8% in MET-treated patients (P = 0.018)	[64]

¹Relative risk of any fracture interpreted as OR with 95%CI for several variables in the population of Denmark (National Health Registry, year 2000); ²Multivariate HR for the development of any new fracture among 1964 Rochester, MN, United States residents after recognition of diabetes mellitus in 1970-1994; ³Exposure for at least 36 mo to hypoglycemic treatments in case subjects and matched control subjects, interpreted as OR with 95%CI; ⁴P < 0.01 for comparison of fracture risk in women with rosiglitazone (unadjusted, contingency χ^2 test); ⁵Percentage of change in BMD at week 80, comparing RSG/MET vs MET. RSG: Rosiglitazone; MET: Metformin; GLY: Glyburide; PIO: Pioglitazone; BMD: Bone mineral density; OR: Odds ratio; HR: Hazard ratios.

metformin twice a day, and the other 100 mg sitagliptin once a day, for 12 wk. In the metformin-treated group, serum ALP and urinary D-piridinoline (DPD) were not significantly different from baseline; conversely in the sitagliptin group, serum ALP and urinary DPD decreased significantly after 12 wk, although BMD was unchanged in both groups.

The effects of pioglitazone and metformin on circulating sclerostin (an osteocyte-derived osteoblast proliferation inhibitor), and biochemical markers of bone turnover were studied in 71 men with T2DM. This group as a whole showed higher serum sclerostin levels than healthy controls. Sclerostin levels were further increased in the sub-set of patients that were treated with pioglitazone, who also showed an increase in serum CTX. On the contrary, metformin-treated patients vs healthy controls showed significantly lower sclerostin levels and unchanged CTX levels^[64]. Although sclerostin is a well-established inhibitor of bone formation, recent evidence indicates that it can also promote osteoclastogenesis by stimulating RANKL produced by osteocytes^[65], suggesting that pioglitazone could increase bone resorption while decreasing bone formation, and the opposite would occur with metformin.

Finally, metformin was tested for bone-defect healing purposes in a clinical study, adding this biguanide to

platelet-rich fibrin in order to treat intrabony defects in patients with chronic periodontitis. The study was designed to evaluate the efficacy of platelet-rich fibrin, 1% metformin gel, or platelet-rich fibrin plus 1% metformin gel, in all cases with open flap debridement, for treatment of intrabony defects in 120 patients with chronic periodontitis. The group treated with platelet-rich fibrin plus 1% metformin gel showed the greatest improvements in clinical parameters, with an increase in percentage radiographic defect depth reduction when compared to metformin alone, platelet-rich fibrin alone or open flap debridement alone^[66].

CONCLUSION AND PERSPECTIVES

Patients with long-term T1 DM and T2 DM can develop skeletal complications or “diabetic osteopathy”. These include osteopenia, osteoporosis and an increased incidence of low-stress fractures. In this context, it is important to evaluate whether current anti-diabetic treatments can secondarily affect bone metabolism. Over the past 10 years, many investigators have studied the effects of metformin on bone, providing a considerable body of pre-clinical (*in vitro*, *ex vivo* and *in vivo*) as well as clinical evidence for an anabolic action of metformin on bone. However three reports (one *in vitro*, one *in vivo*, one clinical) have been unable to link

metformin treatment with bone anabolic processes, underscoring the differences that exist between experimental models in pre-clinical studies, and the low statistical potency inherent in clinical reports that include a relatively small number of patients. In this sense, two caveats should be kept in mind when considering metformin treatment for a patient with T2DM at risk for diabetic osteopathy. In the first place, metformin should probably not be considered an anti-osteoporotic drug; it is an insulin sensitizer with proven macrovascular benefits that can secondarily improve bone metabolism in the context of DM. Secondly, we are still awaiting the results of randomized placebo-controlled studies in humans that evaluate the effects of metformin on bone metabolism as a primary endpoint.

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