

Review Article

Advanced glycation and glycoxidation end products in bone

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ABSTRACT

Hyperglycemia and oxidative stress, enhanced in diabetes and aging, result in excessive accumulation of advanced glycation and glycoxidation end products (AGEs/AGEs) in bone. AGEs/AGEs are considered to be “the missing link” in explaining increased skeletal fragility with diabetes, aging, and osteoporosis where increased fracture risk cannot be solely explained by bone mass and/or fall incidences. AGEs/AGEs disrupt bone turnover and deteriorate bone quality through alterations of organic matrix (collagen and non-collagenous proteins), mineral, and water content. AGEs and AGEs are also associated with bone fragility in other conditions such as Alzheimer's disease, circadian rhythm disruption, and cancer. This review explains how AGEs and AGEs accumulate in bone and impact bone quality and bone fracture, and how AGEs/AGEs are being targeted in preclinical and clinical investigations for inhibition or removal, and for prediction and management of diabetic, osteoporotic and insufficiency fractures.

1. Introduction

Increased propensity of fragility fractures is linked to conditions beyond osteoporosis, such as aging [1,2] and diabetes [3–5]. For example, both type 1 diabetes (T1D) and type 2 diabetes (T2D) increase fracture risk that cannot be solely explained by changes in bone mineral density (BMD) and fall incidences [3,6,7]. Insufficiency fractures, on the other hand, are caused by habitual daily loading in the elderly or in patients with bone fragility [8,9], and are also associated with osteoporosis, osteogenesis imperfecta, and diabetes [10,11]. To understand bone fragility in these conditions, changes in bone matrix quality need to be investigated.

For example, non-enzymatic glycation (NEG) (its classical pathway is also known as the Maillard reaction), a post-translational modification of proteins by sugars, is associated with diabetes and linked to an increase in bone fracture risk [12,13]. It is known that enhanced hyperglycemia in diabetes induces excessive accumulation of advanced glycation end products (AGEs) – the products of NEG – in bone [14,15], including but not limited to pentosidine (PEN) and carboxymethyllysine (CML). The formation of CML and PEN is accelerated by increased oxidative stress due to overproduction of reactive oxygen species (ROS). Therefore, CML and PEN are also referred to as advanced glycoxidation end products (AGEs) [16]. However, not all AGEs are

AGEs, and the proportion of AGEs and AGEs may vary with the extent of hyperglycemia and oxidative stress. Various measures of NEG are reviewed in the next section.

Here, with a focus on diabetes and bone, we sought to review the role of AGEs/AGEs in bone metabolism, bone matrix quality (such as alterations in organic matrix, mineral, and water content), and relate such changes to bone biomechanical properties. We also provide a clinical perspective on the use of circulating measurements of AGEs/AGEs in diabetic population for predicting and managing bone fracture risk. This is a promising avenue to translate the work on AGEs/AGEs in bone from bench to bedside. Furthermore, the review highlights the relationship between AGEs/AGEs and bone fragility beyond diabetes. We discuss the role AGEs/AGEs in skeletal fragility associated with aging, osteoporosis, and other conditions such as Alzheimer's disease, circadian rhythm disruption, cancer, and osteogenesis imperfecta.

2. Formation and identification of AGEs/AGEs in bone matrix

In bone matrix, AGEs/AGEs can be formed via different pathways. Maillard reaction, or the classical pathway starts with the process where an aldehyde form of a given sugar such as glucose reacts with ϵ -amino group of lysine or hydroxylysine to form a Schiff base. The rearrangement of the Schiff base generates diverse Amadori products, which goes

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through further rearrangements, cleavage and covalent binding to form irreversible and stable adducts or crosslinks as known as AGEs [17]. In addition to the Maillard reaction, oxidative stress plays an important role in the formation of diverse sugar degradation products such as glyoxal (precursor for CML, glyoxal-derived lysyl dimer (GOLD), *etc.*), 3-deoxyglucosone (precursor for CML, PEN, pyrraline, *etc.*), and methylglyoxal (precursor for carboxyethyl-lysine (CEL), methylglyoxal-derived hydroimidazolone (MG-H1), *etc.*) [18]. In these alternate glycoxidative pathways, these reactive carbonyl groups react with lysine or arginine to form AGEs/AGEs [19–21].

AGEs/AGEs are used as an umbrella term for a large group of complex chemical compounds (estimated at ~750 types). The identities and chemical structures of many AGEs/AGEs are still not known. Collectively in skeletal tissue, a subgroup of nonspecific fluorescent AGEs/AGEs (including PEN, vesperlysines, crosslines, *etc.*) can be assessed by their total fluorescence content, quantified at 360/460 nm excitation/emission and compared against the quinine standard and normalized to the collagen content [22]. Individually, PEN and CML are the two representative AGEs/AGEs that can be directly measured in bone tissue.

PEN is a fluorescent crosslinking AGE that forms between lysine and arginine residues [23]. It is the first specific AGE that was linked to bone fragility [12], and has been widely used as a direct measurement of AGEs/AGEs in bone. Higher level of PEN in bone matrix has been noted with increased age [24,25], hip fractures [26,27], T1D [14], and T2D [15,28]. In clinical studies, PEN measured in the circulatory system

has been linked to increased fracture risk in osteoporosis [29,30], T2D [12,31] and chronic liver disease [32].

In contrast to PEN, CML is a non-fluorescent and non-crosslinking side-chain modification on the lysine residuals that has been recently identified, isolated and measured in bone matrix [21]. CML is 40–100 times more abundant than PEN in bone tissue [21]. As the most commonly used glycoxidative biomarker in long-lived proteins [33] that captures both the aforementioned classical and alternate pathways to achieve glycoxidation [34–36], CML can be considered to be a potential alternative glycoxidative marker and a dominant AGE in diabetic bone. Indeed, CML accumulation is higher in T2D bone than in healthy control bone [37], and greater levels of CML, measured in serum, are linked to T2D fracture risk [13].

Other AGEs/AGEs, such as glucosepane [38], MG-H1 [20], and CEL [20], have been recently quantified in bone, but their contributions to bone fragility are not yet established. This is an area of active and future investigation. The techniques for identification and measurement of AGEs/AGEs in bone including analytical biochemistry (chromatography [39], mass spectroscopy [20], ELISA [21]) and vibrational spectroscopy (Raman [38,40] and FTIR spectroscopy [41,42]) have been reviewed by Sroga *et al.* [16].

The physiological and pathophysiological range of AGEs/AGEs is not fully established. AGEs/AGEs accumulation is significantly higher in trabecular bone than in cortical bone due to its higher surface-to-volume ratio that allows more access for NEG to occur through bone surface [43]. Consequently, we recommend that, where possible, AGEs/

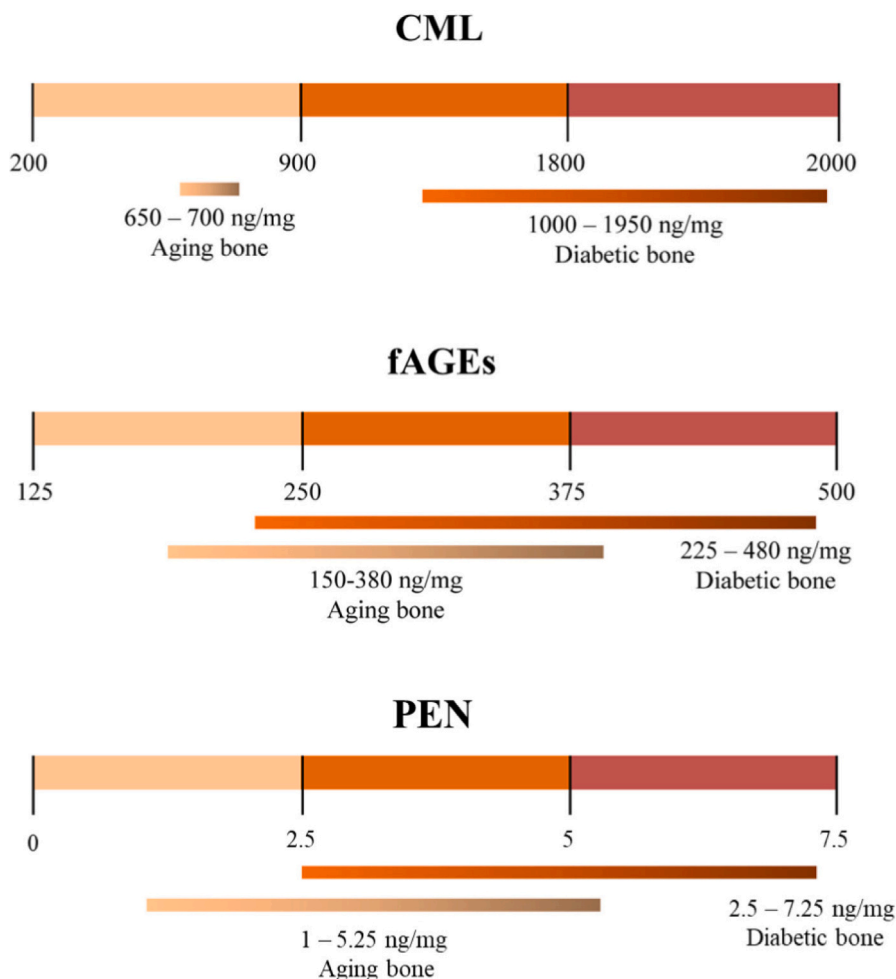


Fig. 1. Pathophysiological and age-related levels of CML [21,37,47], fAGEs [48,49], and PEN [21,28,43]. Figure adapted from Sroga and Vashishth [45].

AGEs should be measured separately in cortical and trabecular bone. Gender differences of AGEs/AGEs accumulation in bone have not been widely explored, with one aging study reporting no significant gender-specific influence [44]. Again, this is a promising avenue for future investigation as AGEs/AGEs impact bone turnover and postmenopausal osteoporosis involves significant changes in bone turnover that can be impacted by accumulated AGEs/AGEs (see next section for further details).

For mechanistic studies, the formation of AGEs/AGEs in bone can be induced *via in vitro* glycation. *In vitro* models of NEG in bone allow to isolate and investigate contributions of AGEs/AGEs on bone material with a short incubation period that enhances the AGEs/AGEs to pathophysiological levels, shown in Fig. 1. Ribose incubation supports the pathway to the formation of PEN, and has shown to be more effective in increasing the level of PEN and total fluorescent AGEs (fAGEs) compared to glucose incubation [23]. In contrast, glyoxal incubation supports enhanced formation of CML over other AGEs/AGEs [45]. As the formation of various AGEs/AGEs share the similar and spontaneous non-enzymatic pathways, dominant formation of a specific AGEs/AGEs requires careful selection and optimization of incubation time, incubation conditions, type and amount of bone [23,45,46], such that AGEs/AGEs form within the pathophysiological range associated with aging and diabetes (Fig. 1).

3. AGEs/AGEs impact on bone metabolism and bone quality

3.1. Bone metabolism

AGEs/AGEs have been known to attenuate bone metabolism by suppressing bone formation and resorption. With the increase of the receptor of AGEs (RAGE) expression, *in vitro* studies showed the AGE-RAGE axis with its inflammation-enhancing activities is the major factor resulting in decreased bone formation by reducing osteoblastic differentiation and proliferation, downregulating matrix production, and increasing osteoblast apoptosis [50–52].

In context of bone resorption, *in vitro* studies show that with augmented RAGE signaling, AGEs decrease the RANKL expression and osteoclastic differentiation [53,54]. Furthermore, *in vitro* cell study demonstrates that the dramatic decrease in RANK expression and the corresponding decrease in osteoclast-like cells resorption ability occurred with increased AGEs concentration at the early cell fusion stage; whereas in the mature stage, there was no effect of AGEs in RANK expression and a slight increase of resorption area was found in one of the AGE concentrations [55]. Since RANK signaling negatively impacts the osteoclast maturity and osteoblast-osteoclast crosstalk [56,57], increased AGEs would decrease bone resorption. In support of the above statement, *in vitro* bone resorption experiments report decreased osteoclastic bone resorption of devitalized bone tissue with higher AGEs content, even in the absence of osteocytes and cell signaling pathways [54,58].

However, when instead of bone slices, *in vitro* glycated bone particles were subjected to *in vitro* resorption in mouse bone cell cultures or *in vivo* resorption *via* subcutaneously in rats glycated bone was more readily resorbed [59]. Similarly, when human bone with naturally occurring levels of AGEs was subjected to *in vitro* resorption using mouse osteoclasts [60], a trend towards increase in bone resorption area and resorption pit density was observed in elderly but not in young donors. Differences between the studies could result from differences in use of bone slices *vs* particles and differences in levels on AGEs. Also, *in vitro* studies often fail to control variations in other bone matrix constituents including but not limited to non-collagenous proteins such as osteopontin that vary with tissue age [61] and control osteoclast attachment and detachment to bone matrix impacting the amount of bone resorption [62]. Thus, in our view spatially matched bone slices where AGEs have been selectively enhanced within the pathophysiological range (see Fig. 1) serve as better substrate/model to investigate the impact of

AGEs on bone resorption *in vitro*.

Given the mixed evidence from the *in vitro* studies, analysis of *ex vivo* human cortical bone containing natural levels of AGEs and resorption pits at time of donor death can be instructive. Such an analysis shows that increase in *in vivo* levels of AGEs is indeed associated with decrease of average *in vivo* resorption pit area as well as percent resorption area (indicating reduced bone resorption), independently of age [63]. This decrease in the resorption ability could be mechanistically explained by the low solubility and digestibility of AGEs/AGEs by enzymes such as collagenase, as demonstrated for other tissues [64,65]. Similarly, other studies using *in vivo* animal models treated with bisphosphonates established negative correlation between PEN and activation frequency [66], as well as between total fluorescent AGEs and the number of labeled osteons [67] (both activation frequency and labeled osteon number are measures of bone turnover).

Decrease in bone formation with altered resorption could lead to altered remodeling and potentially explain low turnover as seen in T2D bone [68]. Such changes results in poor bone quality, accumulation of microcracks [69] and further increase in AGEs [66,67]. Investigating the impact of enhanced AGEs on bone quality in diabetes as well as in postmenopausal and disuse osteoporosis, where bone remodeling processes are significantly altered, is a promising area for future investigations.

3.2. Organic matrix quality

Among the post-translational modifications in bone collagen, enzymatic crosslinks, such as pyridinoline and deoxypyridinoline, stabilize bone collagen arrangement and improve bone mechanical properties [70]. Conversely, accumulation of AGEs/AGEs impairs bone toughening mechanisms. The accumulation of crosslinking AGEs/AGEs on collagen increases the stiffness of collagen matrix [22], reduces the post-yield deformation [71,72], decreases and alters morphology of microdamage [73,74], and leads to loss of energy dissipation [71,72,74] and post-yield toughness [25,72] in bone. At the level of collagen, *in vitro* glycation reduces interfibrillar energy dissipation. At the level of bone tissue, *in vitro* NEG of bone alters bone's toughening mechanism by reducing density of microcracks, and increasing the length of crack segment and uncracked ligaments, making it easy to propagate a crack at a low energy threshold and cause catastrophic failure under impact at 3000–4000 μ strain [74]. These results highlight the role AGEs/AGEs in potentially causing insufficiency fragility fractures during physiological activities [8,9,75]. Similar to *in vitro* results, a T2D rat model, where total fluorescent AGEs were 27 % higher than control, showed a 40 % reduction in ultimate collagen fibril strain indicating weakened ability for collagen to deform and dissipate energy at nanoscale [76].

In contrast to PEN and total fluorescent AGEs, that crosslink bone collagen, the role of CML, a dominant AGE in bone, in causing bone fragility has not been explored. It has been recently proposed that CML could alter the charge distribution of collagen [45], leading to a disruption of molecular organizations within the organic matrix, and thereby impair energy dissipation mechanisms (Fig. 2). However, this mechanism needs to be tested experimentally in future studies.

Other than collagen, glycation also impacts non-collagenous proteins, specifically osteocalcin (OC) – a structural non-collagenous protein [77,78]. Using multiple mass spectroscopy fragmentation techniques our group showed that, during NEG, Amadori products are formed solely on osteocalcin's N-terminus [79]. Thus, glycation is likely to interfere with OC's interaction with collagen and osteopontin and consequently alter nanoscale energy dissipation mechanism in bone, including dilatational bands and diffuse damage that are generated at this scale [77]. Furthermore, a recent ultra-performance liquid chromatography (UPLC) study showed the evidence of *in vivo* modification of OC by PEN, by demonstrating the presence of PEN in the wild-type C57BL/6 mouse tibiae in the chromatogram, while PEN was not detected in mouse tibiae when OC was genetically knocked out. Moreover, *in*

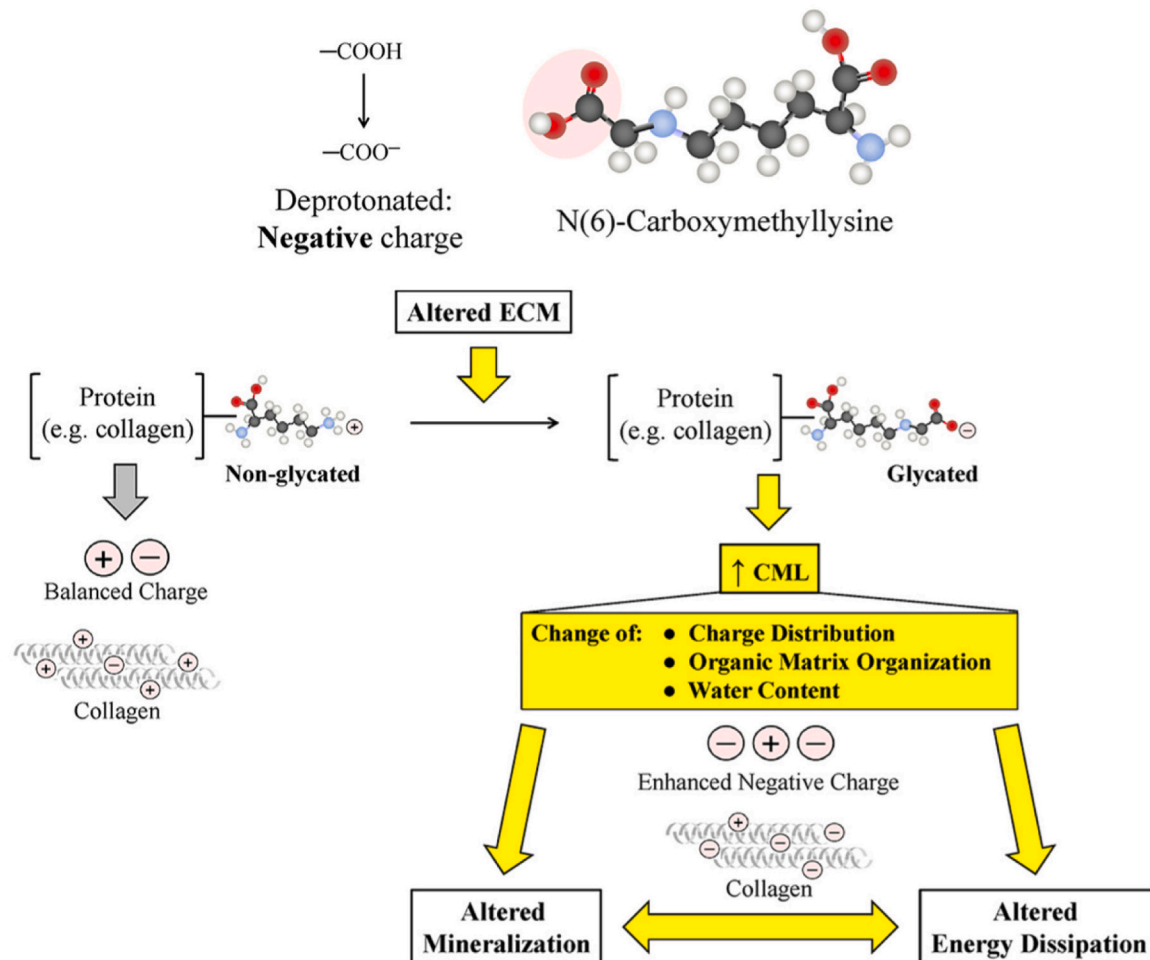


Fig. 2. Proposed mechanisms on CML impacting organic matrix and mineral quality. Figure adapted from Sroga and Vashishth [45]. ECM = extracellular matrix.

vitro glycation, caused a greater increase in total fluorescent AGEs in wild-type bone tissue than in OC deficient bone tissue, and consequently caused a greater decrease of fracture toughness by glycation in wild type bone tissue than in OC deficient mice bones. These results establish that AGEs/AGEs could also modify the structural non-collagenous proteins and impair bone material properties. Moreover, it is suggested that loss of bone toughness during NEG is partially attributable to the negative impact of glycated OC on energy dissipation [80].

3.3. Mineral quality

The mechanisms by which AGEs/AGEs alter mineralization and bone mineral quality are less understood. And there have been inconsistencies in the observations of mineral quality changes that occur in concert with *in vivo* AGEs/AGEs accumulation. For example, a bone biopsy study of T1D patients with fracture history found that the degree of bone mineralization (quantification of bone mineral substance in bone tissue alone that is independent of BMD [81]) was positively correlated to PEN content in trabecular bone, both of which were higher than control [14]. Similarly, for T2D, a non-obese animal model, which eliminates the confounding effect of obesity, demonstrated significantly higher crystallinity and crystal thickness in femora, where the crystallinity was positively correlated with CML and PEN [82]. A study on discarded femoral heads originating from patients who went through arthroplasty due to fragility hip fracture, found a near significant decrease in crystallinity ($p = 0.07$), however, with increased crystal width in T2D group compared to controls. Intriguingly, the mineral-to-

matrix ratio was lower in T2D group compared to controls in this study despite an expected suppression of bone turnover in T2D (not measured). In this study, both control and T2D groups displayed low BMD at around the osteoporotic threshold. This unexpected outcome could be potentially explained by increased AGEs in bone, as the mineral-to-matrix ratio was negatively correlated to total fluorescent AGEs only in the T2D group [42]. However, another study using femora from cadavers showed significant increase of CML as well as higher periosteal mineral-to-matrix ratio in T2D than controls. This study also reported that in a T2D subgroup with high level of cortical porosity, bone tissue had low crystallinity where mineral appeared as non-apatite and poorly crystallized [37].

The above studies report alterations in tissue-level mineral quality as well as the presence and absence of the associations between AGEs/AGEs and various measures of mineralization. However, in order to understand the impact of AGEs/AGEs on bone mineral quality, the mechanisms by which AGEs/AGEs impact mineralization and affect mineral quantity need to be investigated and established. We proposed that enhanced CML may be involved in the mineralization process and may therefore play a role in determining bone mineral quality [45]. The negatively charged carboxyl group in CML tends to attract the positively charged calcium ions which may lead to enhanced mineralization *albeit* in an immature and imperfect non-apatite form. Therefore, acting as a 'molecular link', CML could bridge collagen and hydroxyapatite, and also alter energy dissipation mechanism through its involvement in the mineralization process [45] (Fig. 2). Consistent with this hypothesis we find that in a preclinical mouse model of non-obese T2D, increased CML

formation is indeed positively correlated with higher crystallinity (associated with elongated mineral crystal length [83]), supporting the role of this negatively charged side chain to attract calcium ions, promote growth of hydroxyapatite, and build a physical link between mineral and collagen [82]. Overall, direct literature evidence on the effect of AGEs/AGEs on mineral growth and mineral quality is relatively scant.

3.4. Bound water content

In aging and diabetes, loss of bound water and NEG have been suggested as concurrent processes in collagenous tissues [84]. As a surrogate measure for bone quality and an important contributor to fracture resistance of bone tissue [85–87], the relationship between AGEs/AGEs accumulation in bone and bound water content has been a subject of investigation. One *in vitro* glycation study showed ribose treatment significantly reduced the bound water content in bone which might coincide with the changes in secondary structure of collagen and perturb the hydrogen bonding ability [88]. Similarly, an *in vivo* model of aging showed that, compared to the young group, cortical bone in the aged group consistently showed a significant increase in PEN with a decrease in bound water [89,90]. Subsequent work by the same group suggests that the mechanistic basis of bound water loss, in the above model, is likely another post-translational modification such as collagen deamidation, as BALB/c mice did not show age-related changes in non-crosslinking AGEs. However, studies with diabetic mouse models, such as streptozotocin induced T1D, do show loss of bound water [91] and abnormal collagen crosslinking [92]. In theory, CML accumulation in bone could enhance the hydrophilicity due to the negative charge from its carbonyl group [45], however, the net impact to attract or repel water will depend on all AGEs types. Future studies should evaluate the alteration in bound water content with selective enhancement of various AGEs/AGEs including but not limited to CML.

4. Impact of AGEs/AGEs on mechanical properties and use of agents to inhibit or cleave AGEs and rescue bone fragility

The direct impact of AGEs/AGEs on mechanical properties of bone related to fracture is difficult to evaluate, because under the conditions of aging, osteoporosis, diabetes, and other conditions, several other bone quality parameters are altered. To this end, *in vitro* protocols to induce formation of AGEs/AGEs have been developed to study their direct impact on bone. For example, one of the first studies, developed a dose-response curve wherein machined bovine bone specimens were exposed to ribosylation to dial in specific AGEs/AGEs based on incubation time [22]. Subsequent applications of this approach focused on 7 days of incubation period that doubles the AGEs/AGEs in human cortical and cancellous bone and mimicked the accumulation of AGEs/AGEs within the pathophysiological shown in Fig. 1 [71,72,74,93,94]. Because bone is known to accumulate cracks and not fracture [95], these studies have focused on measurement of post-yield properties rather than energy required to initiate a crack. Results show that enhanced formation of total fluorescent AGEs and/or PEN negatively impact biomechanical properties of bone including reduced propagation fracture toughness (the slope of R curve) [74], post-yield strain [93], damage fraction [71,93], etc. Increased levels of AGEs/AGEs predict various measures of bone mechanical properties associated with human bone fragility [74,94]. Furthermore, mechanisms of reduced energy dissipation at multiple length scales, cited earlier in this review, including increased stiffness of collagen matrix, altered microdamage morphology (transition from self-limiting and efficient form of microcracking (diffuse damage) [75,96] to dominant and fracture prone linear crack and reduction in magnitude of microcracking [73]), uncracked crack bridging [74], etc. were demonstrated due to formation and accumulation of AGEs/AGEs within the pathophysiological range.

In contrast to the above, one recent study on human bone reported

no change [97] in the mechanical properties, in fall-related or quasi-static loading rates, following 4 weeks of incubation in 0.1 M ribose concentration resulting in supraphysiological levels of PEN [>20 mmol/mol of collagen or 2540 % increase over PBS incubated group] in combination with a modest increase in total fluorescent AGEs below the diabetic threshold [200–250 total fluorescent AGEs/mol of collagen]. The inconsistencies in fracture toughness data could be attributed to the different protocols used for *in vitro* ribosylation as the level of total fluorescent AGEs in the group treated with ribose did not reach the diabetic threshold while the pentosidine levels, which represent a small fraction of AGEs/AGEs in bone, exceeded the diabetic threshold > 3 times. Another *in vitro* glycation study similarly found no difference in J-integral fracture toughness between control and glycated samples under fall-like situation, while the reduction in plastic contribution of J-integral, damage fraction, post-yield strain, post-yield energy by *in vitro* glycation could be demonstrated under quasi-static loading rate [72].

On the contrary, a fracture toughness testing approach to capture the tendency of bone to resist the propagation of preexisting cracks, which accumulate with aging [95] and in diabetes [98], measures the changes in crack growth resistance. The results showed that distinctive effect of *in vitro* glycation, corresponding to doubling of fAGEs or three decades of *in vivo* aging, are manifested only in crack growth toughness and not at initiation [74]. Furthermore, under impact loading specimens *in vitro* glycated specimens, corresponding to three decades of aging, failed at 42 % lower strain in proximity of 3000 μ strain that are seen during intense physiological activities [74]. As mentioned in this review, the *in vitro* incubation method using glyoxal to selectively form CML in bone has been established [45]. Future *in vitro* studies could, therefore, focus on including conditions to form CML that allow investigation of the impact of glycoxidative processes in bone.

To address the limitations associated with *in vitro* methods, an alternative approach has been to focus on the relationship between AGEs/AGEs and bone fracture properties in *in vivo* diabetic animal models. For example, in a T1D mouse model, strong negative correlations were determined between propagation toughness with Raman-assessed CML ($R = -0.99$) and PEN ($R = -0.97$) [40]. Similarly, in the aforementioned non-obese T2D model [82], the increase of total fluorescent AGEs explained the loss in cracking toughness and work-to-fracture, and found inverse relationships between Raman-assessed CML and maximum toughness, and between Raman-assessed PEN and toughening effect (difference between initiation and propagation toughness) [82]. A diet-induced T2D mouse model also showed negative correlations between maximum toughness, and total fluorescent AGEs/Raman-assessed AGEs [99].

More importantly, the study demonstrated the rescue of T2D bone skeletal fragility through cleavage of non-enzymatic crosslinks using *in vitro* treatment of phenacyl thiazolium chloride (PTC). The incubation of diabetic bone in PTC reduced total fluorescent AGEs content by 41 % and increased the maximum toughness of PTC-treated samples by 35 % compared to the untreated diabetic bones [99]. Interestingly, similar to *in vitro* ribosylation studies showing accumulation of AGEs/AGEs and a consequent loss bone fracture properties, *in vitro* removal of total fluorescent AGEs and a consequent decrease in bone fragility demonstrate the causality between AGEs/AGEs and bone fragility. Although not in context of diabetes, *in vivo* treatment of mice with chronic kidney diseases with PTC has been shown to decrease bone AGEs [100]. *In vivo* PTC treatment of the diabetic BKS Cg-m+/+ Leprdb mice significantly reduced serum CML [101]. Taken together these results suggest that PTC may have several mechanisms of action. Future work is needed to determine if PTC could both inhibit and cleave AGEs.

In contrast to PTC, pyridoxamine acts as a post-Amadori inhibitor in the formation of AGEs and inhibits the formation of CML on protein during lipid peroxidation reactions [102]. Thus, PM has proven to be a potent inhibitor of AGE formation from glycated proteins [102,103]. A recent study [103] used an *in vitro* model to demonstrate that PM inhibited formation on AGEs and improved parameters associated with

crack growth resistance in bone. These results suggest that PM can indeed prevent loss of bone's mechanical properties. It is noteworthy that PM has been clinically tested in randomized, double-blind, placebo-controlled, multi-center Phase III studies [104]. Future work needs to be done to determine if PM can inhibit formation of AGEs *in vivo*.

Evaluation of the role of AGEs/AGEs in *ex vivo* human bone studies remains challenging, as the bone mechanical properties are also likely to be affected by other heterogeneous confounding factors. In one of the studies, T2D trabecular bone, obtained from individuals undergoing total hip replacement, demonstrated significant inverse correlations between total fluorescent AGEs and toughness, total fluorescent AGEs and post-yield energy, as well as FTIR-assessed nonenzymatic crosslinks ratio and post-yield strain [42]. Negative correlation between trabecular bone total fluorescent AGEs and post-yield displacement have also been reported [105]. Similarly in multiple regression models, PEN and total fluorescent AGEs have been shown to explain the variance in trabecular bone post-yield toughness (adjusted $R^2 = 0.74$) [28]. However, the post-yield mechanical properties in these two studies were not different between T2D group and age-matched controls [28,105]. These studies are based on trabecular bone core assessment, where the mechanical testing is impacted by trabecular bone quantity and microarchitecture, and the post-yield mechanical behavior from compression test is subjected to more variation as the fracture point is not straightforward to determine compared to bending or tensile tests. Also, the duration of diabetes is an important variable that is not available for such *ex vivo* studies. To investigate the relationship of AGEs/AGEs and fracture properties in *ex vivo* T2D bone tissue, future studies should place the emphasis on cortical bone tissue, diabetes duration and material-level mechanical assessments.

5. Clinical implications of AGEs/AGEs in fracture risk

Circulating AGEs/AGEs levels reflect systemic measures of glycation and glycooxidation and these have been correlated to bone AGEs/AGEs [105]. Investigations involving such measures not only contribute to the expanded mechanistic linkage between AGEs/AGEs and bone fractures in large clinical cohorts but are of potential value to address the fracture risk underestimation in diabetes.

In the general population, not specifically related to diabetes, circulating PEN [29,30,106–108] and CML [109] have been separately shown to be associated with the risk of osteoporotic fractures. However, lack of significance in the association with fracture risk following adjustments was also reported in other studies for PEN [110] and CML [106], potentially due to the differences in population (ethnicities and lifestyles) and adjustment methods. In contrast, studies on diabetic fracture risk are more consistent. In Health Aging, Body and Composition (Health ABC) study, urinary PEN [12] and serum CML [13] have separately shown significant associations with the risk of incident clinical fractures specifically in the T2D cohort, while no such relationship was shown in the non-diabetic cohort. Urinary PEN is correlated with increased prevalence of vertebral fractures in T2D population [12] and this relationship persists following adjustment of BMD. Similar findings have been reported in a Japanese study where serum PEN is associated with vertebral fracture risk in diabetic women [31]. Moreover, a negative correlation between urinary PEN and trabecular bone score (measure of trabecular bone microarchitecture) was reported only in the T2D group [111]. Because urinary PEN was not different between T2D and non-diabetic group while serum CML was significantly higher in T2D than non-diabetic group in the Health ABC cohort, one can hypothesize that the pathway by which circulating PEN and CML impact fracture risk may be different, and CML could provide a more specific measure of bone fragility in the diabetic population. Nonetheless, both circulating AGE markers have shown clear utility in evaluation of fracture risk particularly in T2D populations.

Alternatively, a simpler and more universal measurement, glycated hemoglobin (HbA1c), commonly and universally measured *via* simple

blood test, could potentially serve as a surrogate measure for AGEs/AGEs in bone and be used in diabetic fracture risk assessment. In particular, a recent study found a significant positive correlation between trabecular bone AGEs and serum HbA1c levels [42]. Additionally, in a postmenopausal T2D cohort, the long-term HbA1c level was found to inversely correlated with bone material strength index (BMSi, an *in vivo* assessment of bone material-level properties) [112]. A recent review [113] suggests that, compared to other biochemical markers such as bone turnover markers, HbA1c seems to be the only reliable predictor for diabetic fracture risk.

Consistent with the above results and the notions that NEG is a diffusion based process and long-term HbA1c measure may indeed capture both the impact of hyperglycemia and its duration, we evaluated various longitudinal HbA1c averages in large T2D population-based cohort study and found that longitudinal average of HbA1c, measured over two years, is indeed significantly associated with subsequent two-year fracture, where T2D individuals with poor glycemic control (longitudinal HbA1c $\geq 9\%$) had a 29 % increase in adjusted fracture risk compared to T2D individuals with adequate glycemic control (longitudinal HbA1c between 6 % and 9 %) [114]. With adjustments of diabetic comorbidities (retinopathy, neuropathy, nephropathy, cardiovascular diseases, etc.) as well as osteoporosis, the increased fracture risk comparing poor glycemic control against adequate glycemic control in T2D was attenuated to 18 % but remained significant. The remaining relationship adjusted for comorbidities more directly reflects the negative impact of glycation on bone fractures [114]. These findings open new avenues for using glycemic control over a moderate period of time, which potentially would capture the impact of AGEs/AGEs in T2D bone, to address the underestimation of fracture risk in T2D by BMD.

6. AGEs and AGEs beyond diabetes

Beyond diabetes, AGEs/AGEs increase in bone with age [43], and the elevated AGEs/AGEs levels are associated with weaker bone properties including toughness [115]. As mentioned earlier, increased concentration of AGEs/AGEs in bone (PEN in particular) is associated with bone loss in non-diabetic individuals [44], which potentially links AGEs/AGEs in bone to the prevalence of osteoporosis. Indeed, higher levels of circulating PEN and CML have been identified in patients with osteoporosis compared to healthy subjects [116]. Similar to human data, an animal study also found an increased pentosidine level in bone in the ovariectomized model compared to the sham group [117]. Moreover, excessive accumulation of total fluorescent AGEs and PEN in bone was found in a microgravity-induced osteoporosis animal model, where AGEs/AGEs explained the deteriorated trabecular bone microarchitecture and disrupted bone metabolism [118]. Moreover, the inhibition of AGEs/AGEs using irbesartan prevented bone loss due to microgravity and rescued bone fragility [119]. In addition, in two studies related to a cohort of Japanese postmenopausal women, higher levels of bone PEN were discovered in individuals with hip fracture than in non-fractured individuals for cortical bone with lower degree of mineralization [26], and for trabecular bone with higher degree of mineralization [27].

Recent evidence has revealed that AGEs/AGEs explain bone fragility in other pathological conditions. For example, consistent with the notion of disruption of glucose metabolism in Alzheimer's diseases [120], an animal study demonstrated significant associations of weaker bone fracture properties with increased AGEs/AGEs accumulation in bone in a transgenic 5XFAD model of Alzheimer's disease [121]. Similar results were also found under a combination of circadian rhythm and high-fat diet [122]. In metastatic bone diseases, the total fluorescent AGEs accumulation in human vertebral bone was found to be associated with lower vertebral strength and stiffness [123]. Additionally, in an osteogenesis imperfecta mouse model, where bones exhibit brittle fracture properties, a recent study found a 30 % increase in the total fluorescent AGEs accumulation compared to healthy controls [124].

Taken together these studies highlight that AGEs/AGEs may ubiquitously form and impact bone tissues in diseases and conditions involving alterations of hyperglycemia and/or oxidative stress.

7. Summary

In this review, we summarized the bone quality alterations at multiple scales due to the accumulation of AGEs/AGEs. Although the deleterious effects of non-enzymatic crosslinks on collagen and non-collagenous proteins are known, the roles of non-crosslinking AGEs/AGEs, such as CML on diabetic bone quality and related bone mechanical integrity are not yet fully established and require further investigations. Clinical measurements of systemic glycoxidation (circulating levels of PEN and CML) measures have been found to associate with diabetic fracture risk and these results provide new avenues to improve diabetic fracture risk prediction and to better manage and understand bone fragility as an important comorbidity of diabetes. The increased accumulation of AGEs/AGEs in bone is being identified in diseases such as Alzheimer's disease, cancer, osteogenesis imperfecta *etc.* and these results highlight the ubiquitous role that AGEs/AGEs play in the heightened bone fragility under a variety of situations involving alterations in hyperglycemia and/or oxidative stress. Future studies should consider exploring the role of AGEs/AGEs such as CML on bone fragility, gender effect on accumulation of AGEs/AGEs in bone and for use of circulating or surrogate markers of AGEs/AGEs in bone (such as longitudinal glycemic control) to assess and predict fracture risk. The preventive therapeutics for inhibiting and/or removing existing AGEs/AGEs from aging and diabetic bone tissue need to be developed to better manage bone fragility under myriad conditions including but not limited to diabetes, osteoporosis, Alzheimer's disease, circadian rhythm disruption, osteogenesis imperfecta, cancer *etc.*

CRediT authorship contribution statement

Bowen Wang: Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis, Conceptualization. **Deepak Vashishth:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

None.

Data availability

No data was used for the research described in the article.

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