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An improved linear systems model of hydrothermal isometric tension testing to aid in assessing bone collagen quality: Effects of ribation and type-2 diabetes

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Abstract

This study sought to further develop and validate a previously proposed physics-based model that maps denaturation kinetics from differential scanning calorimetry (DSC) to the isometric tension generated during hydrothermal isometric tension (HIT) testing of collagenous tissues. The primary objectives of this study were to verify and validate two physics-based model parameters: α, which indicates the amount of instantaneous isometric tension developed per unit of collagen denaturation, and β, which captures the proportionality between temperature and the generated isometric tension post denaturation initiation. These parameters were used as measures of bone collagen quality, employing data from HIT and DSC testing of human bone collagen from two previous studies. Additionally, given the physical basis of the model, the study aimed to further validate Max. Slope, the rate of change in isometric tensile stress with change in temperature, as an independent measure of collagen network connectivity. Max.Slope has previously been positively correlated with measures of cortical bone fracture resistance. Towards this verification and validation, the hypotheses were a) that α would correlate strongly with HIT denaturation temperature, T_d, and the enthalpy of melting (ΔH) from DSC, and b) that β would correlate positively and strongly with Max.Slope.

The model was employed in the analysis of HIT-DSC data from the testing of demineralized bone collagen isolated from cadaveric human femurs in two prior studies. In one study, data

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CRediT authorship contribution statement

Faezeh Iranmanesh: Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Conceptualization. **Daniel Y. Dapaah:** Writing – review & editing, Methodology, Investigation, Data curation. **Jeffry S. Nyman:** Writing – review & editing, Resources, Methodology, Funding acquisition, Data curation. **Thomas L. Willett:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

were collected from HIT-DSC testing of cortical bone collagen from 74 donors. Among them, 38 had a history of type 2 diabetes +/− chronic kidney disease, while the remaining 36 had no history of T2D again with or without CKD. Cortical bone specimens were extracted from the lateral mid-shaft. The second study involved 15 donor femora, with four cortical bone specimens extracted from each. Of these four, two specimens underwent a 4-week incubation in 0.1 M ribose at 37 °C to induce non-enzymatic ribation and advanced glycation endproducts, while the other two served as non-ribated controls.

The examination involved investigating correlations between the model parameters α and β and various measures, such as Max.Slope, Td, ΔH, age, and duration of type 2 diabetes. The results revealed positive correlations between the model parameter β and Max.Slope (r = 0.55–0.58). The parameter α was found to be associated with T_d, but also sensitive to the shape of the HIT curve around T_d resulting in difficulties with variability and interpretation. As a result, while both hypotheses are confirmed, Max.Slope and β are better indicators of bone collagen quality because they are measures of the connectivity or, more generally, the integrity of the bone collagen network.

Keywords

Bone; Collagen; Hydrothermal isometric tension testing; Differential scanning calorimetry; Integrity; Connectivity; Type 2 diabetes; Ribose

1. Introduction

Bone is a hierarchical tissue composed of both inorganic and organic components [1,2]. The inorganic (mineral) phase of the bone, consisting of calcium-deficient, highly substituted hydroxyapatite crystals, is widely understood to be a major contributor to the yield strength and stiffness of bone tissue [3]. The organic phase, on the other hand, plays an important role in the post-yield mechanical properties of bone [4–6]. The organic phase consists of type I collagen [7], which comprises 90 % of the total protein by mass [8], and water, which occupies 15 %–25 % of bone volume [9]. Without these parts of the organic phase, bone is a brittle material. In addition, bone toughness and strength decrease considerably when bone collagen denatures as a result of heating, although its elastic modulus remains relatively constant regardless of collagen denaturation [4]. Zioupos' findings [5] support collagen's role in toughening mechanisms, indicating that bone fragility in aging and disease may be associated with changes in the collagen network's integrity or connectivity. In a more recent study [6], bone collagen network integrity/connectivity measured by thermomechanical methods was associated with cortical bone fracture toughness measures. Collagen enables bone to withstand relatively large deformation after yielding and before failure, especially under tension; this is known as "ductility" [10,11]. Thus, as the primary ingredient of the organic phase of the bone, collagen has been shown to play an important function in the mechanical behaviour of the bone tissue. Its quality is expected to be a key determinant of tissue-level bone quality, reflecting those parts of tissue composition and microstructure that contribute to bone's fracture resistance apart from bone mineral density [12].

Various diseases, such as diabetes and chronic kidney disease (CKD), may affect bone tissue-level mechanical properties and, as a result, a patient's risk of fracture [13–16]. Although type 2 diabetes (T2D) is associated with normal or even high bone mineral density (BMD), the risk of a fracture is increased in T2D patients [13]. As a result, T2D seems to be related to a decrease in fracture resistance that isn't detected in BMD measurements. Structure, density (reflecting the bone mineral content) and bone tissue quality (reflecting the tissue-level fracture resistance) are key determinants of a bone's ability to resist fracture [13]. Diabetes has been postulated to decrease bone tissue quality rather than bone mineral density [13]. Crucial to bone strength and toughness are the intermolecular cross-links that form between neighbouring collagen molecules. These include both enzymatic and non-enzymatic crosslinks [17]. In a study using a rat model of induced T2D, Saito et al. [18] found that despite a lack of change in BMD, there was a gradual reduction in enzymatic cross-links and a significant increase in advanced glycation end products (AGEs), which include non-enzymatic cross-links and other non-crosslinking post-translation modifications. Accumulation of AGEs may also hinder the production of enzymatic crosslinks [19,20]. These findings could explain the substantial decrease in bone strength compared to non-diabetic control groups observed in this study. However, Saito et al. [18] did not include an examination of bone structure parameters such as cortical thickness or section modulus, aspects that could potentially influence the study's conclusions. Regardless, in diabetes, deterioration of enzymatic cross-links and/or increasing levels of AGEs may affect the quality of the bone collagen and thus the bone tissue quality accordingly leading to a corresponding increase in bone fragility, independent of BMD [13].

Based on clinical data, chronic kidney disease (CKD) is also associated with increased fracture risk [15,21,22]. Similar to diabetes, there are no evident changes in mineral content between normal and CKD diseased bone [23], while the mechanical properties of bone at different tissue levels were degraded in a rat model of CKD [23]. According to studies on animal models of progressive CKD [24,25], although changes in bone mechanical properties in CKD occur due to various factors, alterations in collagen cross-linking have been observed in late-stage CKD. These changes include both lower levels of enzymatic cross-links and accumulation of non-enzymatic advanced glycation end-product cross-links [24,25].

An alternative method to investigate the impact of bone collagen on bone quality involves inducing controlled collagen changes using in vitro models like the non-enzymatic ribation model. Prior research [26–30] indicates that exposing bone tissue to non-enzymatic ribation leads to collagen modifications, resulting in altered bone mechanical properties. This ribation model [31] increases non-enzymatic glycation in bone collagen [29,32], stiffening the organic matrix [29,32]. Ribation in vitro has been found to affect bone mechanical behaviour by reducing post-yield properties associated with ductility [29,30], post-yield energy dissipation [33], bone toughness, stiffness loss in individual trabeculae, and the damage fraction of trabecular bone [34]. Interestingly, studies have shown that pretreatment of bone with ribose before exposure to γ-irradiation, a process known to make bone brittle, not only protected the collagen network stability and connectivity but also helped maintain the mechanical properties of the bone tissue, including strength, ductility, toughness, and fatigue life [26–28,35]. This apparent contradiction demonstrates that the mechanisms

by which collagen contributes to tissue-level bone quality are complex and incompletely understood.

Differential scanning calorimetry (DSC) [36] and hydrothermal isometric tension (HIT) testing [37] are among the techniques used to evaluate the quality of bone collagen. However, interpretation of the results has been challenging. In our previous work [38], a physics-based linear systems model was proposed to link the behaviour measured by the two collagen assessment methods, DSC and HIT. The model outputs two parameters, α and β, that help to evaluate collagenous tissue quality. By capturing the proportionality between the integral of the DSC signal and the early stages of the isometric tension in HIT, α reflects the amount of isometric tension generated per unit of collagen denaturation. The parameter, β, on the other hand, captures the generation of additional tension once the denaturation stage is complete (at temperatures above the denaturation (melting) temperature, T_d), modeling the proportionality between temperature, T, and the developed isometric tension according to rubber (thermo)elasticity theory [38]. However, the model and the way it was applied in the earlier work had some limitations [38]. First, the two parameters, α and β, in the earlier version of the model were recognized to depend on the collagen density within the tissue (collagen mass per unit of tissue volume). It is necessary to account for the tissue's collagen density if one wishes to compare the model parameters between different tissue types and/or run correlation analyses on these parameters within a dataset or across multiple datasets. In our previous work [38], the collagen density was not measured. In this study, the model has been applied to two datasets in which the collagen density was measured. In addition, to better model the HIT test results in different cases, a new term ($\boldsymbol{A}$ in Eq. (1)) has been added to account for the initial stress relaxation that happens in the HIT test. Finally, and perhaps most importantly, the model has been applied to two human bone collagen datasets to demonstrate its capability and for its verification and validation. The first dataset includes tissue from T2D donors with and without CKD. The second dataset contains data from human bone specimens that were exposed to the in vitro ribation model [39]; a model that typically results in controlled collagen alterations including increased thermal stability and increased connectivity of the collagen network.

Therefore, we sought to further develop, verify, and validate our model of HIT-DSC testing for the purposes of collagen quality assessment. The primary objectives of this study were to verify and validate the two physics-based model parameters, α and β, as measures of bone collagen quality using data from HIT and DSC testing of human bone collagen from two previous studies. Towards this verification and validation, the hypotheses were a) that α would correlate strongly with HIT denaturation temperature, T_d, and the enthalpy of melting (ΔH) from DSC, and b) that β would correlate positively and strongly with Max. Slope, which has previously been positively correlated with human cortical bone fracture resistance [6]. Additionally, given the physics-based nature of the model, these correlations would serve to further validate Max.Slope as an independent empirical measure of collagen network connectivity.

2. Materials and methods

2.1. Modeling and implementation

In our previous study [38], a linear systems model was used to associate the thermal and thermomechanical behaviour of bone collagen when evaluated by DSC and HIT, respectively. To generate the output HIT signal using the model, the DSC input signal is convolved with an impulse response function, $J(T)$, which is a function of two parameters α and β. See eq.(1). Both the parameters, α and β, which were determined by optimization, demonstrated potential for evaluating collagen quality [38].

$$J(T) = \alpha + \beta \times (T - T_{onset}) \times u(T - T_{onset}) \tag{1}$$

The parameter α determines how much immediate isometric tension is generated per unit of collagen denaturation in the beginning of the HIT test before denaturation (melting) is complete [38]. It is unitless. The parameter β (K^{-1}), accounts for the proportionality between the difference in absolute temperature between the current temperature and the temperature at which collagen starts to denature and isometric tension evolves [38]. T_{onset} marks the onset of denaturation in the DSC endotherm (Fig. 1). Also, the function $u()$ is the unit step function.

Based on dimensional analysis, to be able to compare the model parameters, α and β, between different tissues, the collagen density in the tissue is required [38]. Upon inclusion of the collagen density, and based on the fact that absolute temperature in degrees Kelvin (K) is being used, the parameter α is dimensionless and the parameter β has units of K^{-1}. In the current work, the collagen density was measured for all specimens in the two datasets described below and added to the model allowing better assessment of collagen quality.

Another modification to the model was the addition of a new term to the optimization parameters to account for the initial stress relaxation that happens in some of the HIT tests. See Fig. 2.

With these modifications, the fitness function which is minimized by an optimization algorithm to determine the model parameters [38], is as follows:

$$\begin{aligned} fitness fcn &= \left|\sigma_{HIT} - x_{out}\right| \\ x_{out}(T) &= \rho_{collagen} \cdot \left[\boldsymbol{\alpha} + \boldsymbol{\beta} \cdot (T - T_{onset}) u(T - T_{onset})\right] * x_{in}(T) + \boldsymbol{A}T + \mathrm{B} \end{aligned} \tag{2}$$

In Eq. (2), x_{in} is the DSC signal (in units of W/g), and x_{out} is the HIT signal (in units of stress, such as $N/mm^3 = J/m^3$ or N/m^2). The parameters in bold font are the optimization parameters, including α and β which are the model parameters previously defined, and $\boldsymbol{A}$ accounting for the stress relaxation in the HIT signal. Additionally, the initial prestress applied to each specimen in HIT testing is accounted for by the parameter **B**. Also, $\rho_{collagen}$

is the collagen density in units of g/m^3. In the fitness function defined above, σ_{HIT} denotes the experimental HIT curve.

2.2. Thermal and thermomechanical characterization of human cortical bone collagen specimens

The bone collagen used in this study was from cadaveric human femurs from two previous studies. The first study involved 74 donors of which 38 donors (15 males; age = 74 ± 12 years old and 23 females; age = 68 ± 12 years old) had a history with type 2 diabetes (T2D) with or without CKD whereas the remaining 36 donors (17 males; age = 72 ± 14 years old and 19 females; age = 68 ± 10 years old) had no history of T2D while some had CKD. Cortical bone specimens were cut from the lateral mid-shaft of the femurs.

The second study involved 15 donors (8 males; age = 56 ± 5 years old and 7 females; age = 54 ± 5 years old). From each femur in this study, four cortical bone specimens were extracted from the lateral mid-shaft. Two of these specimens were randomly assigned to a control group (no ribose incubation) with the remaining two specimens assigned to the ribation group. These specimens were incubated in 0.1 M ribose for 4 weeks at 37 °C [39].

The specimens were de-mineralized in 20 % ethylenediaminetetraacetic acid (EDTA) for 5 weeks at 4 °C. Subsequently, they were demineralized in 0.5 M EDTA at room temperature for another two weeks to ensure full de-mineralization was achieved. After demineralization, the HIT and DSC samples were prepared.

For the T2D study, two HIT samples approximately ~20 mm × 2.5 mm × 1.5 mm (length × width × thickness) were cut using a custom-made parallel ultra-sharp razor blade configuration (American Line, Extra Keen single edge blades). Furthermore, two circular disks used as DSC specimens were prepared using a 4-mm diameter cylindrical biopsy punch (Integra, York, PA, USA). The height of each DSC specimen was measured using digital calipers (MasterCraft, Vonore, TN, USA) and this was used to estimate the volume of each specimen as: $Volume\ (mm^3) = \pi \times (2mm)^2 \times height\ of\ sample(mm)$.

For the ribose study, due to a limited availability of de-mineralized cortical bone, only one HIT specimen and one DSC specimen were prepared. The HIT specimens were prepared similarly to the T2D study but had dimensions of ~15 mm × 2.5 mm × 1.5 mm. However, the DSC specimens were prepared differently as cuboids approximately 3 mm in length, 2.5 mm width and 1 mm thickness. The exact dimensions of each DSC specimen were measured using the same digital calipers and the volume of each specimen was estimated as: $Volume\ (mm^3) = length\ (mm) \times width\ (mm) \times thickness\ (mm)$.

All specimens, irrespective of origin, were tested with HIT and DSC according to the same protocol, described below.

2.2.1. Hydrothermal isometric tension testing: A custom-built HIT system which allows six specimens to be tested simultaneously was used. The HIT specimens were tested by clamping them about 3 mm away from each end with custom-made clips. The clips held the specimens under isometric constraint. The constrained specimens were then heated

in a 4 L water bath from room temperature to 90 °C at an average heating rate of 1.4 °C/min. Often, there is an initial stress relaxation in the specimens when heating commences due to the viscoelastic nature of the collagen network. As heating continues, the collagen network denatures (melts) transitioning from a triple helix structure to a random amorphous coil gelatinous structure. The temperature at which this occurs is termed the denaturation temperature (T_d). This transition drives the collagenous tissue to shrink; however, due to the isometric constraint, a tensile stress is generated within the specimen instead. During the test, the temperature as well as the tensile load generated by the specimen are measured and recorded by a resistive temperature device (RTD) and load cell respectively. The tensile load is converted to stress by dividing by the specimen's initial cross-sectional area. From the corresponding stress versus temperature curve, maximum rate of change in isometric tensile stress is denoted as Max.Slope and is a function of the overall connectivity of the collagen network. Also, the difference between the minimum and maximum stress achieved during the test was computed and denoted as Diffstress. Fig. 3 shows a representative HIT test curve indicating Max.Slope, denaturation temperature (T_d), and Diffstress.

2.2.2. Differential scanning calorimetry: The differential scanning calorimetry (DSC) was carried out following the methods reported in previous work [32,40]. The tests were performed using a TA Instruments DSC Q-2000 system (TA Instruments, New Castle, DE, USA). In DSC, the heat flow difference between a specimen placed in a sealed pan and an empty reference pan heated at a constant rate is measured. When the specimen melts, termed denaturation when testing collagen or other proteins, more heat will be absorbed in the sealed pan compared to the reference pan. This is represented as an endotherm when the heat flow difference is plotted against the temperature and characterises the thermal behaviour of the collagen.

Each specimen was placed in a hermetically sealed aluminium pan (TA Instruments, New Castle, DE, USA) and then heated from 25 °C to 95 °C at a constant rate of 1.4 °C/min to match the heating rate of the HIT tests. From each test, a heat flow vs. temperature curve was generated. The curve was then analyzed (Universal Analysis Software, TA Instruments, New Castle, DE, USA) to compute the temperature at the onset of heat flow (T_{onset}), the temperature at maximum recorded heat flow (T_{peak}), the enthalpy of denaturation (ΔH), and the full width at half maximum (FWHM) of the denaturation endotherm. After the test, the DSC specimens were lyophilized and then weighed to obtain the dry mass of each sample. The collagen density of each specimen was computed by dividing the dry mass of each specimen by the volume of each specimen.

The HIT and DSC data from each specimen was then used in the modified linear systems model described in Section 2.1 to generate α and β parameter values for each specimen.

2.3. Statistical analysis

Pearson correlations were tested between α and β and other measures including age, type 2 diabetes duration, Max.Slope, enthalpy of denaturation, Td, and Diffstress.

To identify the explanatory variables that best predict the model parameters, α and β, a stepwise linear model was utilized where the predictor variables were selected in a step-by-

step manner based on their statistical significance and their contribution to the predictive power of the model. This is an automated process that starts with an initial model that includes all the predictor variables, and then iteratively removes or adds variables to the model based on their contribution to the model's fit. Covariates were not included in the stepwise linear model. For example, Diffstress was not included because it is a covariate of Max. Slope.

3. Results

The modified model [38] was applied to the two sets of data. The first set included data from bone collagen specimens from T2D donors and controls. A total of 7 predictors were examined (Table 1), however one of these predictors, namely the duration of T2D, was only applicable to the T2D donor group. For four specimens within this group, the duration of the disease was unknown, leading to their exclusion from this analysis. The HIT measures, Max.Slope ($r = 0.58$, $p < 0.0001$, Fig. 4) and its covariate Diffstress ($r = 0.52$, $p < 0.0001$), correlated well with the parameter β. A weak negative correlation ($r = -0.23$, $p = 0.04$) was also found between the DSC measure, FWHM, and the parameter β. The denaturation temperature, T_d, which quantifies the thermal stability of collagen network, was associated with α ($r = -0.58$, $p < 0.0001$), and a weak correlation was interestingly identified between the duration of type 2 diabetes (T2D duration) and the parameter α ($p = 0.036$). (See Table 2.)

The absolute values of the α parameter for the three groups in the T2D study (T2D, non-T2D, and CKD) are shown in Fig. 5.

In the DSC thermogram, the onset of collagen denaturation is indicated by T_{onset} (Fig. 1) while in the HIT curve, denaturation is detected at T_d (Fig. 3). Due to differences between these two methods and the heterogeneity of the biological tissues, T_{onset} and T_d are often different for each specimen. Nevertheless, there exists a correlation between these two parameters, as depicted in Fig. 6.

Analysis of the ribation study specimens focused on three predictors (Max.Slope, T_d, and Diffstress) because age and T2D duration were not applicable. The results show that, like the first study on type 2 diabetes (Table 1), both Max.Slope ($p < 0.0001$) and Diffstress ($p < 0.0001$) were positively correlated with β. Additionally, a negative correlation was found between T_d, a measure of thermal stability, and α ($p < 0.0001$), which was also observed in the previous study. Interestingly, a new correlation between α and Diffstress was found in this analysis.

A two-tailed *t*-test was conducted to compare the α parameter values between the two groups (0.0 M ribose and 0.1 M ribose). The results, depicted in Fig. 7, indicate a significant reduction ($p < 0.05$) in the α parameter for bone samples that were incubated in 0.1 M ribose. However, the same analysis on the β parameter, didn't detect significant differences in the β parameter between the two groups (Fig. 8).

As mentioned above, a stepwise linear modeling approach was employed to identify statistically significant predictor variables. Since the parameter Diffstress was a covariate

of Max.Slope, and enthalpy was a covariate of T_d in the T2D study, Diffstress was not considered in the stepwise linear regression for both studies, and enthalpy was not considered only for T2D study. As shown in Table 3 and Table 4, T_d was the key contributor to the prediction of α (with $p < 0.0001$ in both studies), while Max.Slope was the primary independent determinant of β (with $p < 0.0001$ in both studies).

Fig. 9 presents example plots overlaying the model output, x_{out}, over the paired DSC (x_{in}) and HIT data to which the model was fit.

4. Discussion

In this study, the model reported by Iranmanesh and Willett [38], has been modified and applied to a broader range of HIT-DSC data from human bone collagen specimens from individuals with and without type-2 diabetes and chronic kidney disease and specimens with and without in vitro ribation (0.0 M Ribose or 0.1 M Ribose) [39]. The objective was to verify and validate the proposed model from Iranmanesh and Willett [38] using a broader human dataset by determining whether the two model parameters α, and β, which are proposed to capture bone collagen quality, correlate with any of the experimentally determined measures in the study such as Max.Slope, T_d, age, duration of the disease, etc. This process involves additional validation of Max. Slope as an independent measure of collagen network connectivity, which has previously been demonstrated to correlate positively with cortical bone fracture toughness. A notable improvement in this study was the incorporation of collagen density into the model initially reported by Iranmanesh and Willett [38]. This enabled us to compare the α and β values across the datasets and conduct correlation analyses, which were not feasible in the previous study. Moreover, our hypotheses were confirmed through correlation analysis, revealing that α is associated with T_d and the enthalpy of denaturation, while β demonstrates a positive correlation with the Max.Slope derived from experimental HIT testing. It is noteworthy that the advantage of the model parameters α and β over T_d and Max.Slope lies in their derivation from the combination of denaturation kinetics (DSC data) and rubber thermoelasticity theory (HIT data), whereas Max.Slope and T_d are solely derived empirically from the HIT data.

In the type 2 diabetes dataset, a meaningful correlation ($r = 0.58$, $p < 0.0001$) was found between the parameter β and Max.Slope over a wide age range and both sexes with and without chronic kidney disease. Max.Slope represents the maximum rate of isometric tension generation after the denaturation temperature in the HIT test, which is a measure of collagen network connectivity. This finding is noteworthy because a previous study by Willett et al. [6] demonstrated a significant correlation between Max.Slope and transverse fracture resistance of human cortical bone, J-int ($R^2 = 35$ %, $p < 0.001$). Also, Ziopous et al. [5] reported a strong correlation between the same measure (maximum rate of load contraction or RF_{max}) and fracture toughness measures ($r = 0.65$ for K_c and $r = 0.86$ for J-integral).

The stepwise linear regression analysis (as presented in Table 3 and Table 4) consistently identified Max.Slope as the primary predictor of the parameter β. These findings suggest that β may serve as a suitable measure for assessing the quality of a collagenous tissue.

The identified correlations between β and Max.Slope not only qualifies β as an indicator of collagen quality but also reaffirms the validity of Max.Slope as an independent measure of collagen network connectivity considering that β is a physics-based model parameter.

Although a higher degree of correlation between β and Max.Slope ($r > 0.8$) might have been anticipated, it is important to recognize that β is derived from both HIT and DSC curves, while Max.Slope is based solely on the HIT curve. Therefore, the scatter of the data in Fig. 4 can be attributed to the variation in the data from these two testing methods. Another contributor to this scatter may be linked to cases where the model output did not entirely fit with the experimental HIT curve, although the average R-square value for the dataset is 0.998. Moreover, inevitable errors, such as errors in specimen mass measurement due to scale sensitivity, and variability in DSC endotherms may introduce uncertainty in the β parameter.

Enthalpy of melting (ΔH) was also identified as a second significant predictor of β in the ribose study. Note that it wasn't considered as a predictor in T2D study. This observation is intriguing because enthalpy is proportional to the area under the DSC thermogram, and, based on the model, was expected to affect α rather than β. In the T2D study, enthalpy correlated with the parameter α, while in the ribose study, it correlated with β. This might be attributable to the differences in the state of the collagen between the two studies. In the T2D study, it's possible that collagen molecules were damaged or denatured due to the disease state, whereas in the ribose study, they have undergone chemical modification by non-enzymatic ribation rather than damage or denaturation.

Regarding the parameter α, both the T2D study and the ribose study revealed a correlation between the parameter α and the denaturation temperature, T_d (T2D: $r = -0.58$, $p < 0.0001$; ribose: $r = -0.495$, $p = 0.0001$). T_d serves as an indication of the thermal stability of the collagen network and is affected by the nativity or crystalline organization of the fibrillar structure of the collagen [6]. Thus, the observed correlation establishes α as a metric for assessing collagen quality. In addition, based on the stepwise linear regression (Table 3 and Table 4), T_d made a significant contribution in predicting α. The physical meaning of α is associated to the tension resulting from the abrupt increase in conformational entropy as collagen denatures [38]. Therefore, the correlation between α and T_d not only supports α as a nativity metric but also aligns with the underlying physical understanding of α.

Following the detection of the correlation between α and T_d, a *t*-test was conducted to compare the α values between the two ribose groups (Fig. 7). Interestingly, the α values were significantly different between the two ribose study groups consistent with the findings of Unal et al. [39]. According to the study by Unal et al. [39], a significant difference in T_{onset} values between the two ribose groups was detected, leading to the conclusion that ribation increases the thermal stability of the collagen network perhaps by stabilizing the intrafibrillar structure. It is important to highlight that in the study by Unal et al. [39], T_{onset} was significantly higher in the 0.1 M ribose group, whereas in this study, α is notably higher in the non-ribated group. This is explained by the negative correlation found between α and T_d and the positive correlation found between T_d and T_{onset}, both of which capture the thermal stability of the collagen network. Also, β values were not detectably different

between the two ribose groups (Fig. 8), consistent with the findings of Unal et al. [39], where no significant difference was reported in the Max. Slope between the two ribose groups.

As mentioned, T_{onset} indicates the onset of denaturation in a DSC thermogram, while T_d signifies the initiation of collagen melting in an HIT curve. Although a slight difference between these two temperatures is anticipated due to the difference between these two methods, a strong correlation was anticipated between these parameters. According to Fig. 6, despite a notable correlation between the two measures T_{onset} and T_d, the observed correlation in the T2D study is not as strong due to the scatter of the data being higher. One possible explanation for this observation is higher heterogeneity in T2D specimens, evident in other observations such as Fig. 5 where the standard deviation of parameter α is significantly larger within the diabetic group compared to other groups.

In the T2D study, a weak correlation was found between α and T2D duration. However, no correlation was found between age, α and β. Therefore, it seems that additional research is needed to explore the relation between the T2D duration and the model parameters. Interestingly, some recent studies have suggested that bone collagen is relatively disorganized at sites of remodeling of fracture calluses in animal models of pre-diabetic obesity [41]. Additionally, when applying the model to the HIT-DSC data from the T2D specimens, it was intriguing to find that the α parameter in the T2D study had negative values whereas α was positive in the ribation study. After careful examination of the data and model fits, one explanation is that stress relaxation was more significant in many T2D cases around T_d (refer to Fig. 9-A) leading to an artifact in the model fit. This requires further investigations to understand if it is artifactual or not. The correlations of β with enthalpy of denaturation (ΔH) also require further investigation because, according to the model [38], only α should depend upon ΔH as α acts to magnify the integration of the DSC curve (ΔH) to fit the early behaviour of the HIT curve.

In previous studies [6,39,40], the two parameters T_d and Max.Slope, both obtained from the HIT curve, were utilized as indicators of collagen network stability and connectivity, respectively. With the identified correlations between β and Max.Slope, as well as α with T_d, over a wide range of human bone collagen data, it appears that these parameters (α and β) are physically meaningful, and hold promise as suitable measures for assessing the nativity, organization, and connectivity of the collagen network. Since α and β are derived through the mapping of DSC to the HIT curve via convolution, they potentially offer a more meaningful approach for evaluating collagenous tissue quality within the limitations of the measurements and methods used.

This study has its limitations. Given that the two model parameters, α and β, are potential parameters for assessing collagenous tissue quality, including bone tissue, their efficacy should be validated through additional studies designed to relate them to tissue mechanical properties. Therefore, it is essential to establish correlations between these parameters and measures of tissue quality, such as stiffness, strength, fracture toughness and other relevant measures. Additionally, it's worth noting that, in certain cases, the model may not fully match the experimental HIT curve such as in proximity to Td in the T2D specimens noted

above. Further refinements to the model may be necessary in the future to enhance its accuracy and fit to the experimental data.

In conclusion, we have demonstrated the use of our modified linear systems model for the evaluation of collagenous tissue quality, specifically for human bone collagen in this case, based on paired HIT and DSC data. By shedding light on the significance of collagen and its quality in determining overall bone quality, the study underscores a critical factor that has traditionally been overlooked in bone quality assessments. The confirmation of strong relationships between α and the denaturation temperature, T_d, and between β and the collagen network connectivity, measured as Max.Slope, further verify and validate the model and our physics-based understanding of hydrothermal isometric tension testing.

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Data availability

Data will be made available on request.

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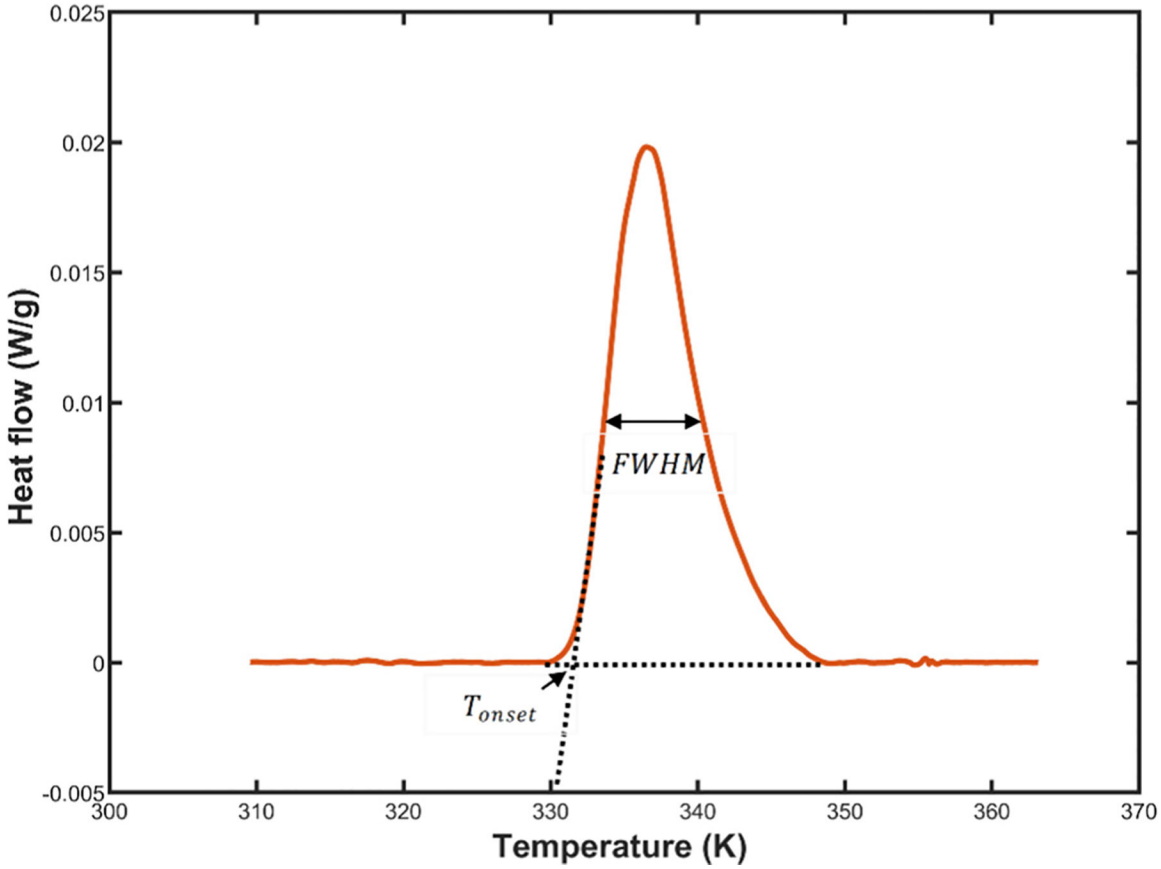

Fig. 1.

A typical differential scanning calorimetry (DSC) endotherm showing the temperature at the onset of detectable melting, T_{onset}. Onset of melting is detected as an increase in absorbed heat. Area under the peak represents the enthalpy of denaturation, ΔH.

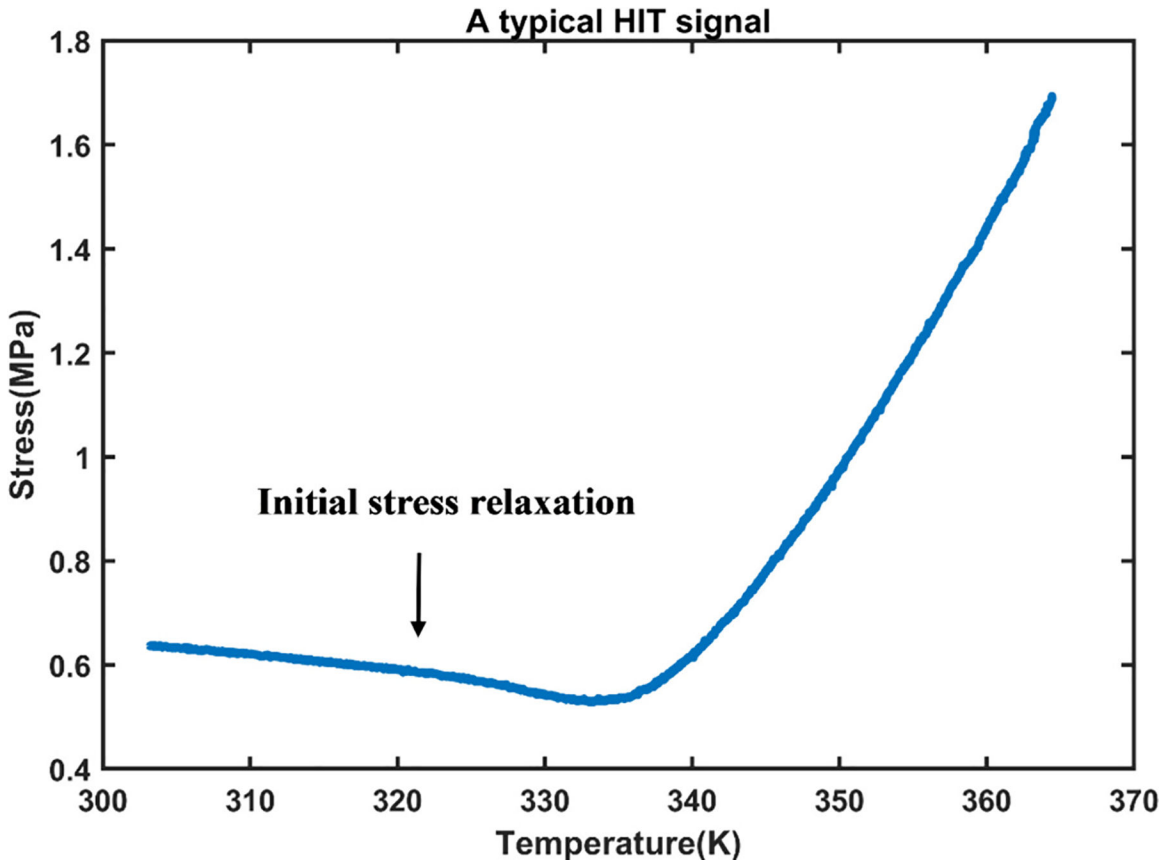

Fig. 2.
Initial stress relaxation observed prior to the onset of denaturation in some HIT tests.

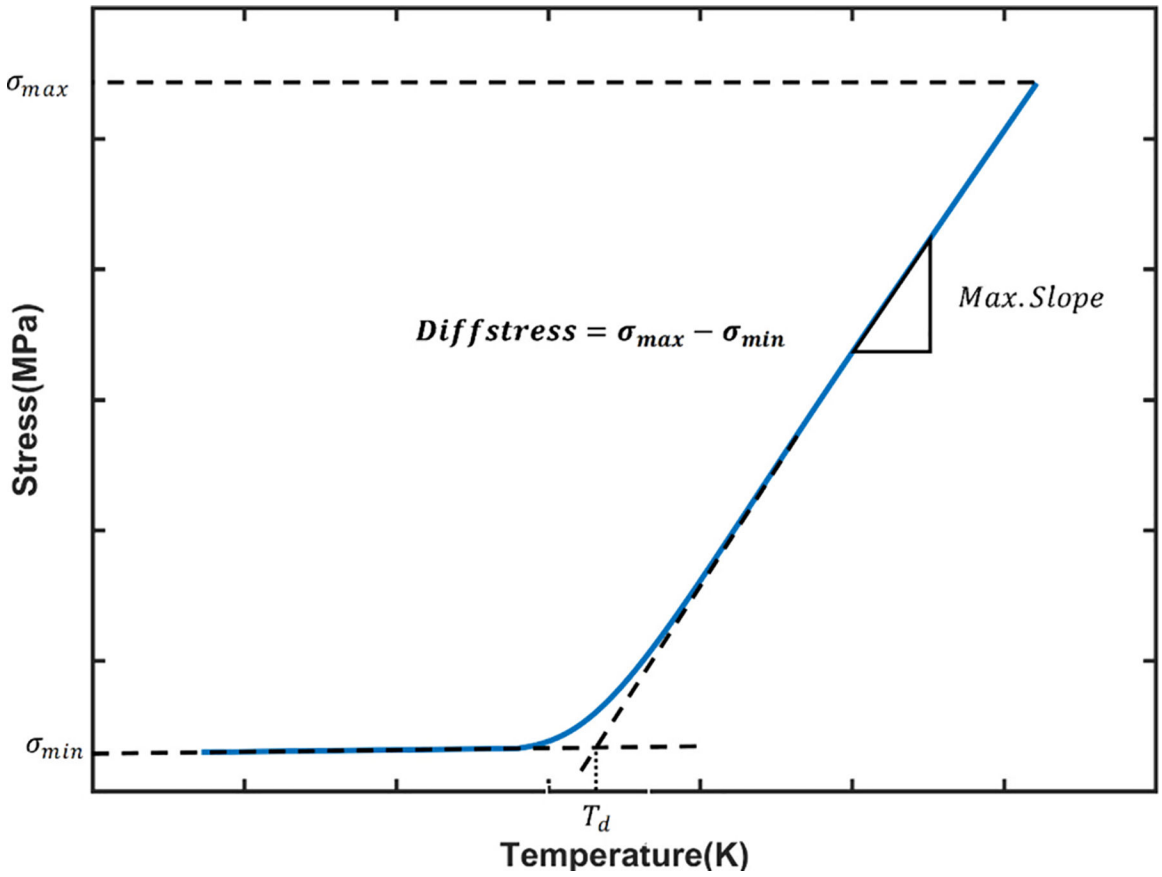

Fig. 3.
The various empirical measures taken from a typical Hydrothermal Isometric Tension (HIT) curve.

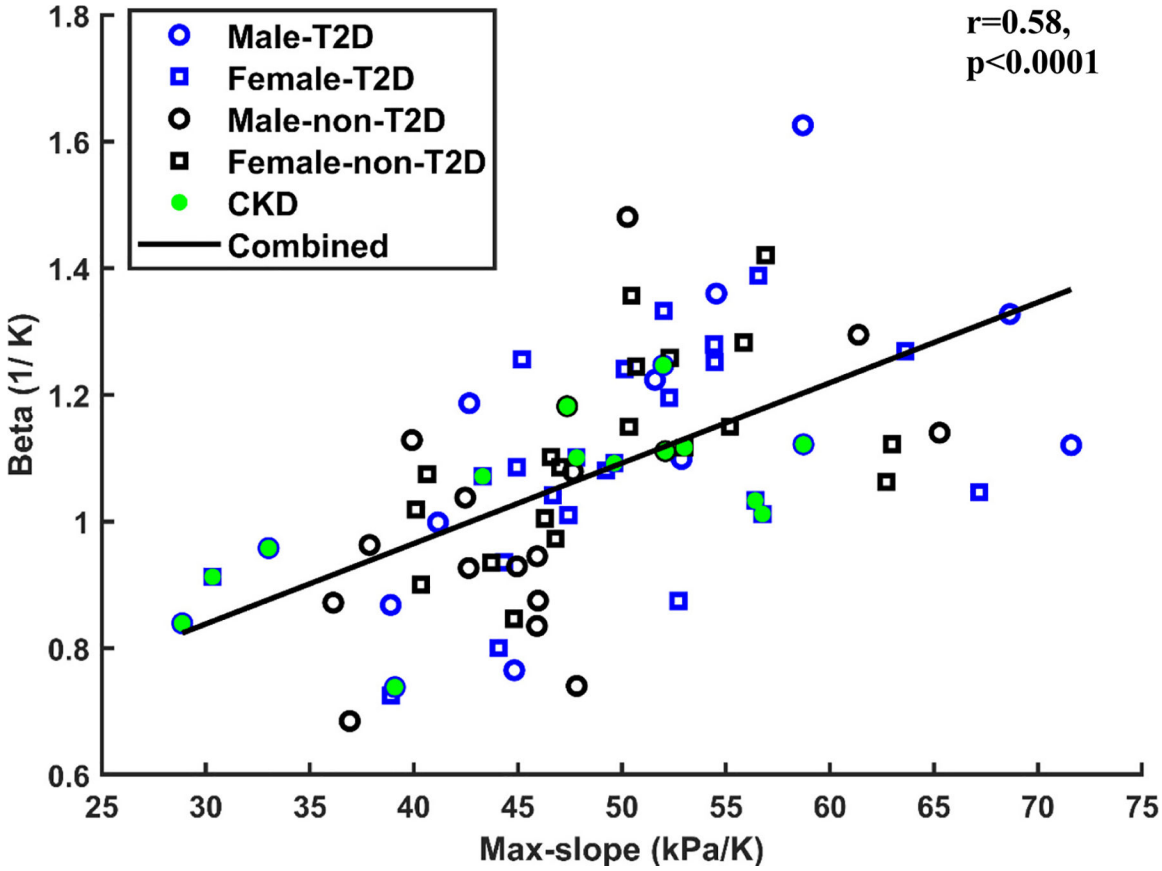

Fig. 4.

β versus collagen connectivity measure, Max.Slope. A relatively strong correlation was found over a wide range of specimens and diseased groups.

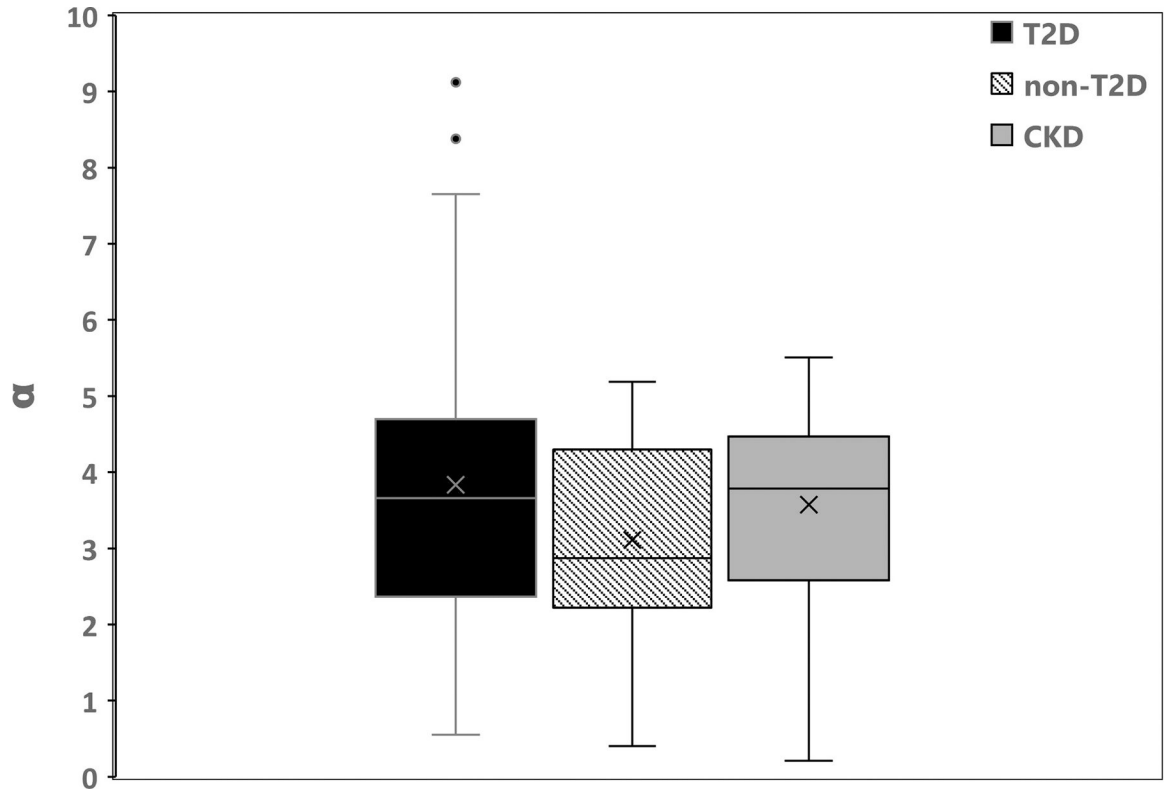

Fig. 5.
Variation of the parameter α between the three groups (T2D, non-T2D, and CKD).

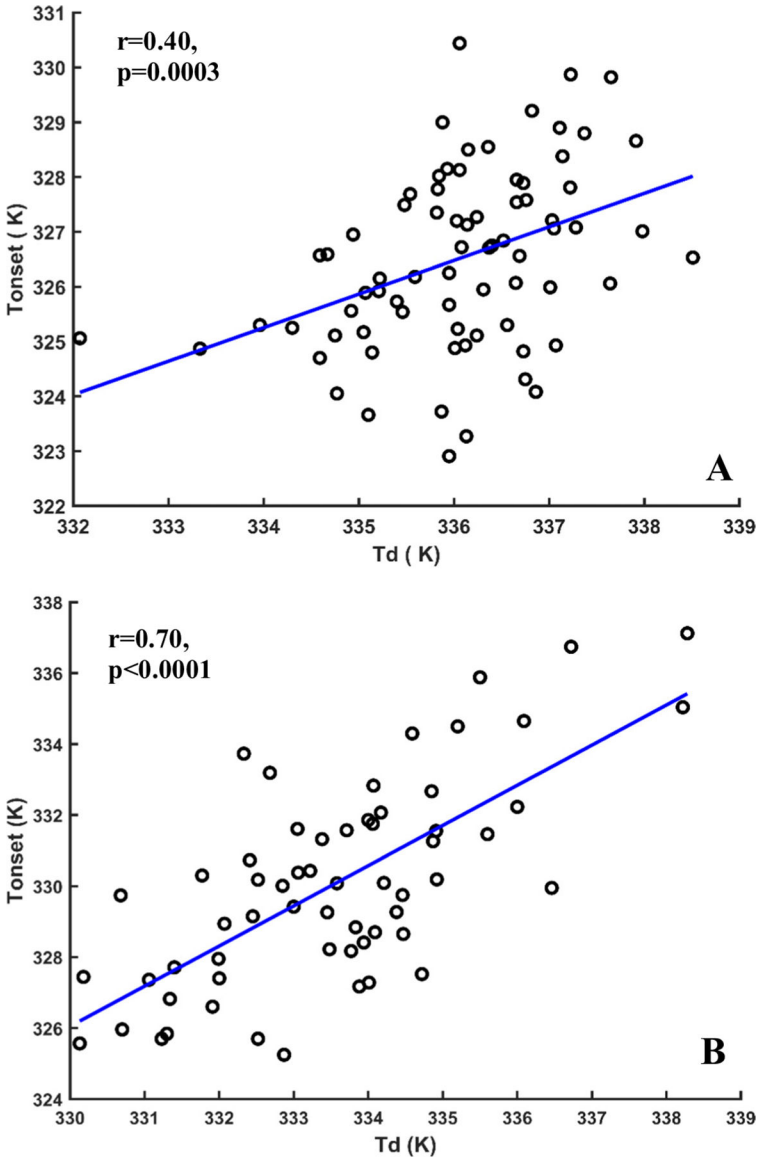

Fig. 6.

Correlation between T_{onset} and T_d in A) the T2D study group and B) the Ribose study group.

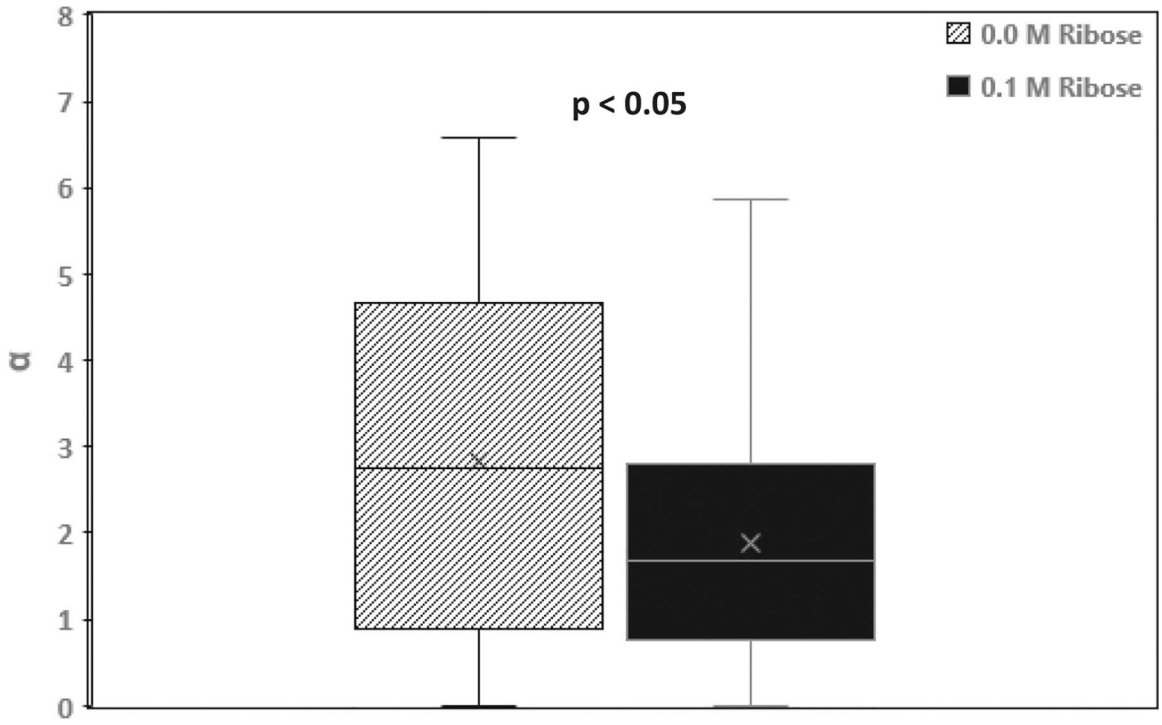

Fig. 7.
The effect of ribose incubation of human bone collagen on the model parameter α.

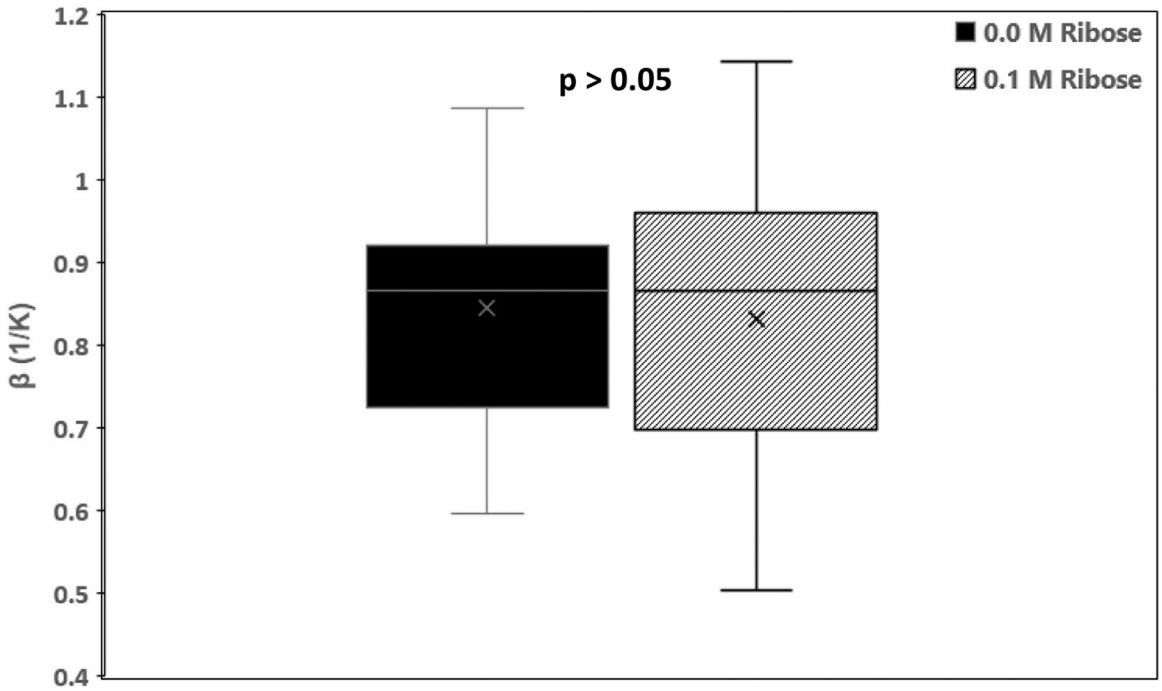

Fig. 8.
The effect of ribose incubation on the model parameter β.

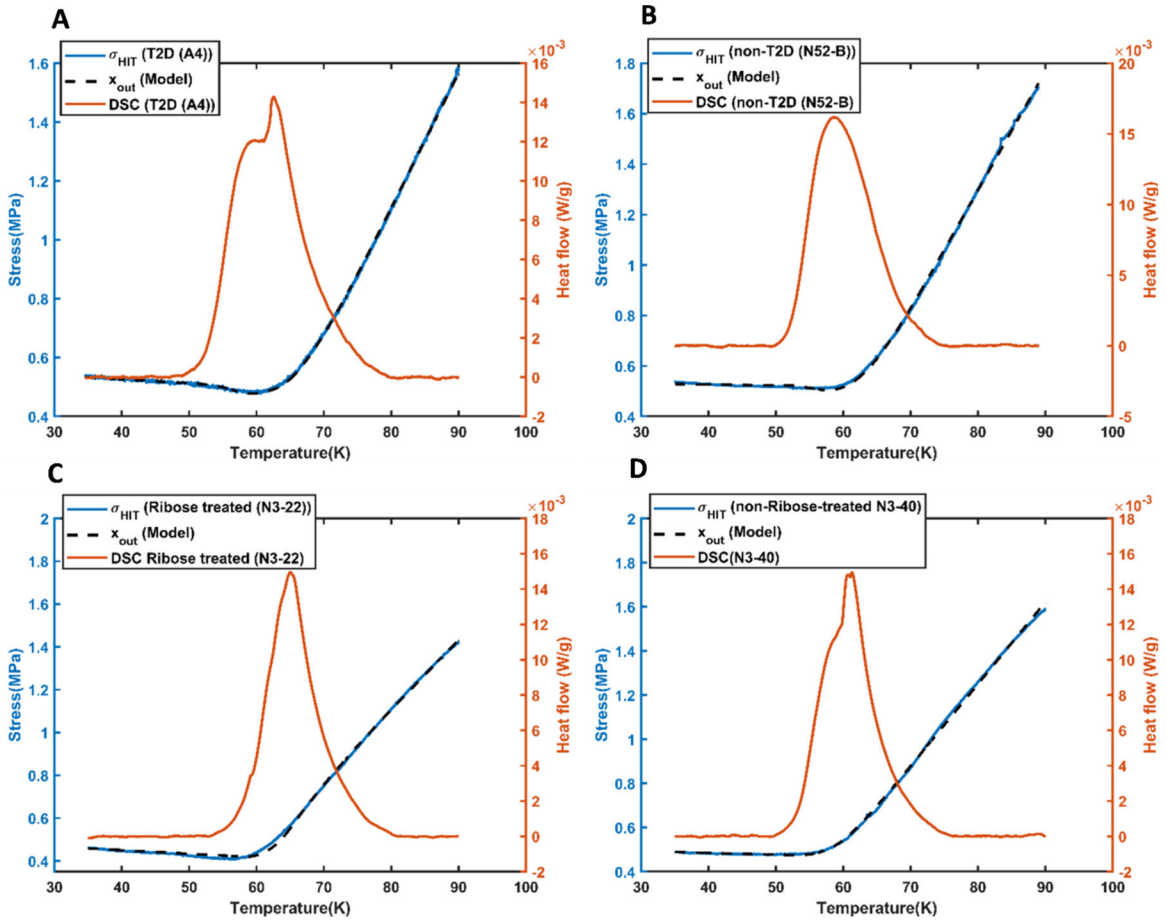

Fig. 9.

DSC and HIT data and the resulting model curve (x_{out}) for four example specimens. A) A T2D specimen, B) a non-diabetic control specimen from the T2D study, C) a ribated specimen, and D) a non-ribated control specimen.

Table 1

Pearson correlation results for α and β versus experimental measures for the Type 2 diabetic (T2D) specimens.

α vs.	***r***	***p*-Value**	**β vs.**	***r***	***p*-Value**
Max.Slope	0.096	0.42	**Max.Slope**	**0.58**	**<0.0001**
Age	−0.022	0.85	**Age**	−0.026	0.83
T2D duration	**0.36**	**0.036**	**T2D duration**	−0.11	0.52
T_d	**−0.58**	**<0.0001**	**T_d**	0.15	0.2
ΔH^*	**0.40**	**0.0004**	**ΔH^***	−0.21	0.07
Diffstress	−0.21	0.07	**Diffstress**	**0.52**	**<0.0001**
FWHM	0.067	0.57	**FWHM**	**−0.23**	**0.04**

Bold font in the table indicates when a correlation was found to be statistically significant ($p<0.05$).

*Enthalpy of denaturation.

Table 2

Pearson correlation results for α and β versus other measures from the ribation study.

α vs.	***r***	***p*-Value**	**β vs.**	***r***	***p*-Value**
Max.Slope	0.074	0.57	**Max.Slope**	**0.55**	**<0.0001**
T_d	**−0.495**	**0.0001**	T_d	0.11	0.41
ΔH^*	−0.02	0.87	**ΔH^***	**−0.47**	**0.0002**
Diffstress	**0.44**	**0.0004**	**Diffstress**	**0.53**	**<0.0001**
FWHM	0.19	0.14	**FWHM**	−0.24	0.06

Bold font in the table indicates when a correlation was found to be statistically significant ($p<0.05$).

*Enthalpy of denaturation.

Table 3

Stepwise linear regression results from the Type-2 diabetes study.

Response variable	Significant Predictor Variables selected by stepwise linear model	R-squared (%)	*p*-Value
α	T_d ($p < 0.0001$) ($R^2 = 33.2$ %)	33.2	<0.0001
β	Max.Slope ($p < 0.0001$) ($R^2 = 33.2$ %) α ($p = 0.0001$) ($R^2 = 0.91$)	45.9	<0.0001

Table 4

Stepwise Linear regression results from the ribose study.

Response variable	Significant Predictor Variables selected by stepwise linear model	R-squared (%)	*p*-Value
α	T_d ($p < 0.0001$) ($R^2 = 24.5$ %)	24.5	<0.0001
β	Max.Slope ($p < 0.0001$) ($R^2 = 30.1$ %) ΔH ($p < 0.0001$) ($R^2 = 22$ %)	58.8	<0.0001