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# The different distribution of enzymatic collagen cross-links found in adult and children bone result in different mechanical behavior of collagen

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ABSTRACT

Enzymatic collagen cross-linking has been shown to play an important role in the macroscopic elastic and plastic deformation of bone across ages. However, its direct contribution to collagen fibril deformation is unknown. The aim of this study is to determine how covalent intermolecular connections from enzymatic collagen cross-links contribute to collagen fibril elastic and plastic deformation of adults and children's bone matrix. We used *ex vivo* data previously obtained from biochemical analysis of children and adults bone samples ($n = 14$; $n = 8$, respectively) to create 22 sample-specific computational models of cross-linked collagen fibrils. By simulating a tensile test for each fibril, we computed the modulus of elasticity (E), ultimate tensile and yield stress ($\sigma_u$ and $\sigma_y$), and elastic, plastic and total work ($W_e$, $W_p$ and $W_{tot}$) for each collagen fibril. We present a novel difference between children and adult bone in the deformation of the collagen phase and suggest a link between collagen fibril scale and macroscale for elastic behavior in children bone under the influence of immature enzymatic cross-links. We show a parametric linear correlation between $W_e$ and immature enzymatic collagen cross-links at the collagen fibril scale in the children population that is similar to the one we found at the macroscale in our previous study. Finally, we suggest the key role of covalent intermolecular connections to stiffness parameters (*e.g.* elastic modulus and $W_e$) in children's collagen fibril and to toughness parameters in adult's collagen fibril, respectively.



## 1. Introduction

During childhood and adolescence, bone structure is altered by geometrical growth associated with increases in mass, and alterations in tissue density [13,61,62]. These processes build a bone with an optimal size, shape, and architecture to withstand the normal physiological loads that might come from children and teenagers' – subadults'— "tendencies to explore, fall over, off and out of things" [15]. Indeed, it has been established that their bone mechanical properties are different to adults' [9–11,13–15,17–18,42] and that might come from both genetic and environmental factors [59,60]. Furthermore, because the mechanical demand on children's bones is higher than in adults, it could be suggested that it is important for them to have a higher macroscopic toughness and stress-dissipation capacity of bones, rather than macroscopic stiffness [15,34].

Both immature (*i.e.* children) and mature (*i.e.* adults) bone tissues are composed mainly of an organic matrix of collagen (*i.e.* tropocollagen molecule — TC) with minerals (*i.e.* carbonated apatite nanoplatelets — cAp) and water. In connective tissues, such as bone and skin, TCs assemble into collagen fibrils and are stabilized by several posttranslational modifications that allow the formation of intermolecular and interfibrillar collagen cross-links (Fig. 1a–b) [6,7,21,70]. The two main kind of collagen cross-links depicted are enzymatic and non-enzymatic. Enzymatic collagen cross-links form intermolecular covalent liaisons between specific amino acids of tropocollagen helices to stabilize collagen fibrils [21,27,48,55,59,60]. They come from a physiological enzymatic pathway where the initial immature form links two collagen molecules together (*i.e.* hydroxylysino-norleucine (HLNL) and dihydroxylysino-norleucine (DHLNL)) (Fig. 1c). With time, immature enzymatic collagen cross-links further react with another collagen molecule to mature into a trivalent form (*i.e.* pyridinoline (PYD) and deoxypyridinoline (DPD)) (Fig. 1c). This conversion can be quantified as enzymatic collagen cross-links maturity which is defined by the ratio of trivalent (*i.e.* mature) and divalent (*i.e.* immature) cross-links

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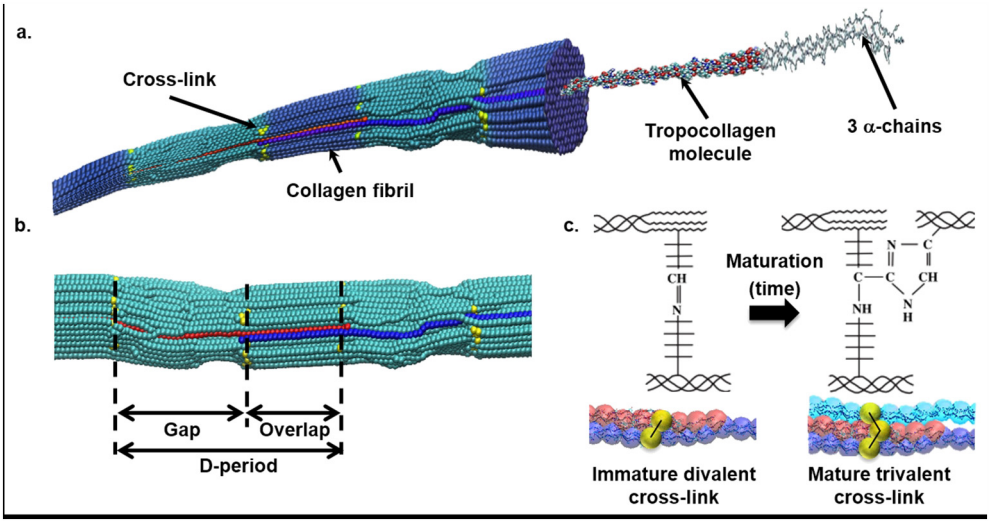


**Fig. 1.** (a) Hierarchical structure of a collagen fibril. (b) Representative section of the molecular model of ca collagen fibril. The characteristic gap and overlap region are clearly visible. (c) Representative forms of both divalent and trivalent cross-links (top) and representation in the molecular model (bottom). Over time, immature divalent cross-links will evolve into the mature trivalent form.

[11,48,57,65]. Enzymatic collagen cross-links can only form in few very specific locations of the terminal domains of the collagen molecule yielding a finite number of possible enzymatic collagen cross-links formation [63] and their density correspond to the amount of intermolecular covalent liaisons per mol. of collagen formed during this process. The chemical reactions leading to the formation of mature enzymatic collagen cross-links is a non-reversible continuous process [21,22,41,59,60,70], and effectively turns the individual tropocollagen molecules into a large interconnected polymer structure. Non-enzymatic collagen cross-links come from Glycation process and are depicted as Advanced Glycation End-products (AGEs). They are associated with aging or pathological conditions (diabetes) and impair collagen matrix normal function [3,31,74]. They can form in multiple locations along the length of the collagen and their density can be significantly larger that physiological enzymatic collagen cross-links [31,55]. Their positioning occurs randomly compared to enzymatic collagen cross-links [66,70], they accumulate with time where tissue turnover is slow [1]. Due to children's high bone turnover, AGEs are present in very few quantities in children's bone [11].

On one hand, TCs are mainly stabilized by intermolecular covalent liaisons from enzymatic collagen cross-links [6–7] to build a soft interconnected matrix [59,60,67]. On the other hand, the mineral phase is made of several cAp that nucleate inside the collagen matrix and link with TC [5,26,28] by several intermolecular forces [20], such as hydrogen or ionic bonds, to reinforce the soft matrix. Although enzymatic collagen cross-links, TC-cAp interface and water molecules have been shown to be determinants of the mechanical properties of bone; results are dependent on species, age, and testing method, making it difficult to understand their specific contributions. Indeed, experimental results on children's bone mechanical properties are very diverse [9,11,16,18,44,50] and there is still a gap of knowledge about the contribution of each bone element to children's bone mechanical behavior. In children population, pathologies affecting collagen such as lathyrism (an alteration of enzymatic collagen cross-links that does not affect the mineral [52]), Menkes disease (a deficient activity of lysyl-oxidase that causes defective in enzymatic collagen cross-linking [37]) and osteogenesis imperfecta (an alteration of collagen structure and enzymatic collagen cross-links that seem to be regulated independently to mineral crystallinity [65]), impact bone biomechanics and leads to pathological fractures. Therefore, understanding bone enzymatic collagen cross-links in children's bone is pivotal to improve both current therapies and diagnosis in children population affected by enzymatic collagen cross-links related bone pathologies.

Although previous results indicate that enzymatic collagen cross-links density in children bone is higher than in adult bone while enzymatic collagen cross-links maturity is lower [11,59,60], the respective contribution of enzymatic collagen cross-links density and maturity to bone deformation remains unclear in the children population. Our previous *ex vivo* mechanical tests of human bone samples have shown that enzymatic collagen cross-links maturity negatively correlates plastic energy dissipated before bone fracture at the macroscopic level. Our hypothesis is that *in silico* mechanical tests of collagen fibril – built from the data of the human bone samples previously tested — will show that enzymatic collagen cross-links maturity correlates with plastic energy dissipated before collagen fibril fracture. Furthermore, we anticipate that the evolution of both enzymatic collagen cross-links density and maturity is a significant contributor to collagen fibril mechanical behavior. To test our hypothesis, we have built 22 computational models from a mesoscale coarse-grained model of collagen fibrils [19] created using sample-specific enzymatic collagen cross-links data ((HLNL + DHLNL) and (PYD + DPD)) taken from biochemical analysis of bone samples of children and adults donors [11]. Collagen fibril mechanical properties (Modulus of Elasticity ($E$), ultimate and yield stress ($\sigma_u$, $\sigma_y$), strain for ultimate and yield stress ($\varepsilon_u$, $\varepsilon_y$) point elastic, plastic and total work ($W_e$, $W_p$ and $W_{tot}$)) have been obtained from computational tensile test to establish the link between collagen fibril elastic and plastic deformation and collagen fibril composition.

## 2. Materials and methods

### 2.1. Biochemical characterization

A total of 22 parallelepiped cortical bone samples (children samples n = 14 and adult samples n = 8) were obtained from fibula bones taken from 10 donors (7 children, ages = 11.6 ± 4.7 years and 3 adults, ages = 74.3 ± 9.9 years). The samples were then prepared to perform biochemical analysis to quantify the amounts of immature (HLNL + DHLNL) and mature (PYD + DPD) collagen cross-links. Details of experimental characterization can be found in our previous study [11].

### 2.2. Molecular model

A geometrically accurate mesoscale molecular model of a collagen fibril has been used in this study, in which a bead-spring model of a single tropocollagen helix has been replicated to form the collagen fibril (MATLAB r2014a, Mathworks Inc., Natick, MA, USA) [19] (Fig. 1). Both

immature and mature enzymatic collagen cross-links are introduced to the model. Sample-specific models are created in which both enzymatic collagen cross-links density and maturity are tailored to the biochemical analysis performed on bone samples. Following this protocol, 22 fibrils have been created to represent the 22 bone samples previously described.

### 2.3. Simulation parameters and data analysis

Numerical mechanical testing has been performed according to the protocol defined by Depalle et al. [19]. The coarse-grained models of single collagen fibrils have been used to simulate a tensile test until fracture at a constant loading velocity of 1 m/s. All the simulations have been performed using the molecular dynamic code LAMMPS ((Large-scale Atomic/Molecular Massively Parallel Simulator) [75] in a canonical ensemble with constant number of particles (N), constant volume (V) and constant temperature (T) in the system (NVT ensemble) with a temperature set to 300 K. The stress-strain curves computed from the simulations have been analyzed according to standard for bone studies [56] to obtain modulus of elasticity (E), ultimate and yield stress ($\sigma_u$, $\sigma_y$), strain at ultimate and yield stress ($\varepsilon_u$ and $\varepsilon_y$) elastic work ($W_e$), plastic work ($W_p$), and total work to failure ($W_{tot}$) which correspond to *in silico* (*i.e.* numerical) energies dissipated before fracture. Failure was set as being the point of $\sigma_u$ for each individual fibril. To calculate $W_e$ and $W_p$, the yield point ($\sigma_y$, $\varepsilon_y$) is obtained by using the 2% offset method (Turner and Burr, 2001). $W_e$ is obtained by measuring the area under the curve from the beginning until $\varepsilon_y$, and $W_p$ from $\varepsilon_y$ until failure of the fibril. $W_{tot}$ is the sum of $W_e$ and $W_p$. The mechanical analysis has been performed using a custom R script [76]. The raw data up to the failure point have been fitted to a fifth-order polynomial to minimize the simulation noise when computing the yield points. In the analysis, the cross-linking state has been analyzed by looking at the number of covalent liaisons per mole of collagen formed during the enzymatic process. A divalent cross-link forms one intermolecular covalent liaison between two collagen molecules and a trivalent cross-link links three molecules with two intermolecular covalent liaisons. Enzymatic collagen cross-links maturity is quantified by the collagen cross-link ratio which is the quotient of the quantity of trivalent (*i.e.* mature) collagen cross-links divided by the quantity of divalent (*i.e.* immature) collagen cross-links for the same collagen fibril. We define a covalent liaison by a direct link between two molecules created by a chain of covalently bonded atoms from an enzymatic cross-link. The quantification of the amount of intermolecular covalent liaisons combine information about both the density and the maturity of the cross-links.

### 2.4. Statistical analysis

Statistical analyses have been performed with SPSS (Version 11, IBM, Armonk, NY). Normal distribution has been assessed with Shapiro-Wilk test; Pearson correlation has been performed in the event of normal distribution, and Spearman correlation in the event of non-normal distribution. Variance has been tested with Levene test. Differences between groups have been evaluated with Student's *t*-test for individual samples in case of normal distribution and with a Welch correction in case of non-equal variance, Mann-Whitney test have been performed in case of non-normal distribution. For all statistical tests, significance has been set at $\alpha = 5\%$.

## 3. Results

Two representative stress-strain curves of a child (15-year-old) and an adult (75-year-old) are shown in Fig. 2. The child fibril exhibits higher maximum stress and yield-point strain than the adult fibril. The mechanical properties computed from the stress-strain curves are summarized in Table 1 and all of them are displayed in Supplemental Table 1. For all numerical models of collagen fibrils, collagen cross-links ratio and density (Supplemental Table 1) are within 5% of the corresponding experimental values [11]. Level of significance of parametric and non-parametric correlations are reported on Supplemental Table 2, and linear coefficient and Spearman's rho are reported accordingly.

Comparing the average collagen fibril mechanical properties of children and adult samples, $W_e$ for collagen fibrils in the children group is significantly higher when compared to the adult group (*t*-test, $p = 0.001$) (Fig. 3). Similarly, E is significantly higher in the children's group than in the adult's group (Mann Whitney test, $p = 0.02$) (Fig. 3). No difference was found for $W_p$, $\sigma_u$ and $\sigma_y$. Regarding the evolution of the mechanical properties through lifetime, we also found a monotonically decreasing relationship between age and $W_e$ (Spearman's $\rho = -0.51$, $p = 0.015$) (Fig. 4). More precisely a monotonically decreasing relationship has been found between age and $W_e$ for the fibrils from the adult's group (Spearman's $\rho = 0.71$, $p = 0.045$) but such relationship has not been found in the children's group.

As depicted in Table 1 for all collagen fibrils ($n = 22$), our results show several significant parametric relationships between both collagen cross-links ratio and intermolecular covalent liaisons, and mechanical parameters related to elasticity. More precisely, for collagen fibril related to the children population ($n = 14$), our results show several significant parametric relationships between divalent enzymatic collagen cross-links, covalent and trivalent, and mechanical parameters related to elasticity such as $\sigma_y$, $W_e$. (Fig. 5a) and E (Fig. 5c). However, for collagen fibril related to the adult population ($n = 8$), our results show several significant parametric relationships between divalent enzymatic collagen cross-links, covalent and trivalent, and mechanical parameters such as $\sigma_u$, $\sigma_y$, $\varepsilon_y$, and parameters related to toughness such as $W_e$, (Fig. 5a) $W_p$ and $W_{tot.}$ (Fig. 5b) with a high level of $R^2$ for all of these relationship (roughly 0.80).

We show a linear correlation between all fibril tested for $W_e$ and intermolecular covalent liaisons and collagen cross-links ratio (Supplemental Table 2) and also a distinct threshold distinguishing between children and adults structure and mechanics: in children, most of the $W_e$ values are above 60 MPa and, collagen cross-links ratio and intermolecular covalent liaisons are above 2 and 1.75 respectively, while in adults, most of the $W_e$ are below 60 MPa and, the collagen cross-links ratio and intermolecular covalent liaisons below 2 and 1.75 respectively (Fig. 5c, d). Thus, our results suggest that collagen fibril with a cross-link ratio above 2 have a greater capacity for collagen fibril elastic deformation.

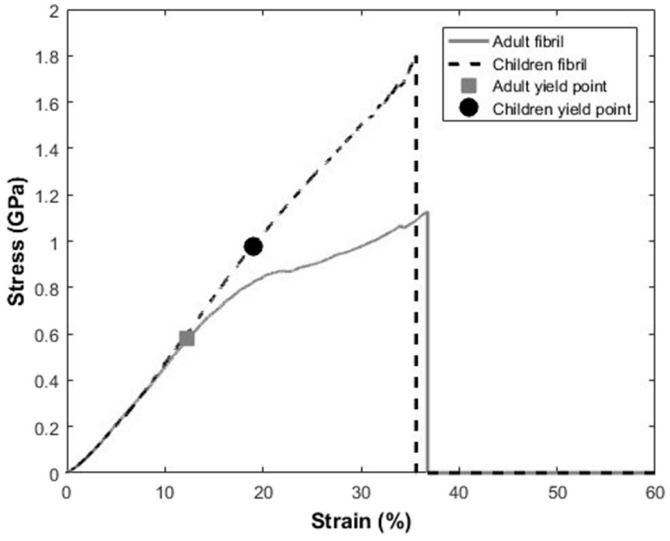


**Fig. 2.** Two representative stress-strain curves of a child (15-year-old) and an adult (75-year-old). The child fibril exhibits higher maximum stress and yield-point strain than the adult fibril.

**Table 1**
Collagen fibril mechanical properties (Modulus of Elasticity (E), maximum and yield stress (σmax σyield) and elastic and plastic ($W_e$ and $W_p$)) obtained from computational tensile test of 22 fibrils that have been created to represent 22 parallelepiped cortical bone samples.

| Collagen fibril | Age (years) | E (Gpa) | $\sigma_u$ (Gpa) | $\sigma_y$ (Gpa) | $W_e$ (Mpa) | $W_p$ (Mpa) | $W_{tot}$ (Mpa) |
|---|---|---|---|---|---|---|---|
| 1 | 5 | 5.92 | 1.97 | 0.97 | 85.10 | 233.10 | 318.20 |
| 2 | 5 | 5.96 | 1.66 | 1.24 | 134.12 | 132.10 | 266.21 |
| 3 | 5 | 6.21 | 1.92 | 1.07 | 98.75 | 222.57 | 321.33 |
| 4 | 7 | 5.61 | 1.69 | 0.80 | 59.23 | 241.52 | 300.76 |
| 5 | 7 | 5.43 | 1.55 | 0.75 | 54.55 | 229.88 | 284.43 |
| 6 | 11 | 5.62 | 1.69 | 0.82 | 63.94 | 227.04 | 290.98 |
| 7 | 11 | 5.88 | 1.53 | 0.99 | 86.80 | 134.33 | 221.13 |
| 8 | 12 | 6.10 | 1.98 | 1.03 | 92.41 | 209.88 | 302.29 |
| 9 | 12 | 5.41 | 1.70 | 0.71 | 48.71 | 219.54 | 268.24 |
| 10 | 15 | 5.90 | 1.95 | 1.12 | 108.87 | 209.76 | 318.64 |
| 11 | 15 | 6.20 | 2.22 | 1.13 | 108.44 | 237.40 | 345.84 |
| 12 | 16 | 5.81 | 1.80 | 0.98 | 86.99 | 228.63 | 315.62 |
| 13 | 16 | 6.09 | 2.09 | 1.10 | 104.73 | 230.69 | 335.42 |
| 14 | 16 | 5.96 | 2.10 | 1.09 | 104.70 | 212.45 | 317.15 |
| Mean | 11 | 5.86 | 1.85 | 0.99 | 88.38 | 212.06 | 300.45 |
| Standard deviation | 4 | 0.26 | 0.21 | 0.16 | 24.42 | 34.78 | 32.49 |
| 15 | 66 | 5.50 | 1.13 | 0.58 | 33.06 | 217.50 | 250.56 |
| 16 | 66 | 5.46 | 1.35 | 0.65 | 42.09 | 227.53 | 269.62 |
| 17 | 66 | 5.30 | 1.44 | 0.68 | 44.84 | 228.12 | 272.96 |
| 18 | 75 | 5.50 | 1.82 | 0.75 | 53.54 | 275.81 | 329.35 |
| 19 | 75 | 5.50 | 1.67 | 0.78 | 58.66 | 229.24 | 287.90 |
| 20 | 75 | 5.75 | 2.09 | 0.91 | 76.32 | 273.64 | 349.96 |
| 21 | 75 | 5.33 | 1.52 | 0.72 | 50.77 | 224.74 | 275.51 |
| 22 | 96 | 5.39 | 1.54 | 0.70 | 48.11 | 227.07 | 275.19 |
| Mean | 74 | 5.36 | 1.53 | 0.72 | 50.93 | 237.96 | 288.88 |
| Standard deviation | 10 | 0.04 | 0.01 | 0.10 | 12.84 | 22.99 | 33.44 |

## 4. Discussion

In this work, we have used coarse-grained molecular modeling to investigate the link between enzymatic collagen cross-links density and maturity, and fibrillar mechanical behavior of bone collagen matrix. We first established a novel difference between children and adult bone in the collagen fibril deformation. We show that in children, a high covalent liaisons number, primarily formed by immature enzymatic cross-links, correlates mainly with elastic deformation parameters (E, $W_e$, $\sigma_y$) while in adults, despite the lower amount of covalent bond liaisons, collagen fibrils which connected with stronger mature cross-links, correlates with energy dissipation and toughness parameters ($W_e$, $W_p$, $W_{tot}$, $\sigma_u$). Our results show that the mechanical response depends on the amount of both divalent and trivalent enzymatic collagen cross-links in adults but only of divalent enzymatic collagen cross-links in children due to the low amount of trivalent. As children bone contains large amounts of immature cross-links (high density) our results show that a high number of interconnected molecules favor the elasticity of the collagen. On the contrary, a lower connectivity but with stronger cross-links (more mature cross-links) as observed in the adult population favors the plasticity of the collagen despite the global lower cross-links density.

However, the amount of intermolecular covalent liaisons is the best factor for predicting collagen fibrils mechanics in both children and adults showing that the intermolecular connectivity formed by cross-linking is the main feature providing high mechanical properties. This suggests a continuous evolution of the mechanical properties of bone with age but more data is necessary to confirm this trend. Our results also suggest a link between collagen fibril and macroscopic elastic behavior in children bone under the influence of immature enzymatic cross-links by showing a parametric linear correlation between $W_e$ and immature enzymatic collagen cross-links at the collagen fibril scale in the children population similar to the one we found at the macroscale in our previous study [11].

While our results show that a cross-linked collagen fibril can reach about 30% deformation (Fig. 2) – which is consistent with previous experimental mechanical testing [64] – our results also show that $W_e$ is dependent on the amount of intermolecular covalent liaisons from enzymatic collagen cross-links and maturity, two factors that are dependent on age. The high amount of immature enzymatic collagen cross-

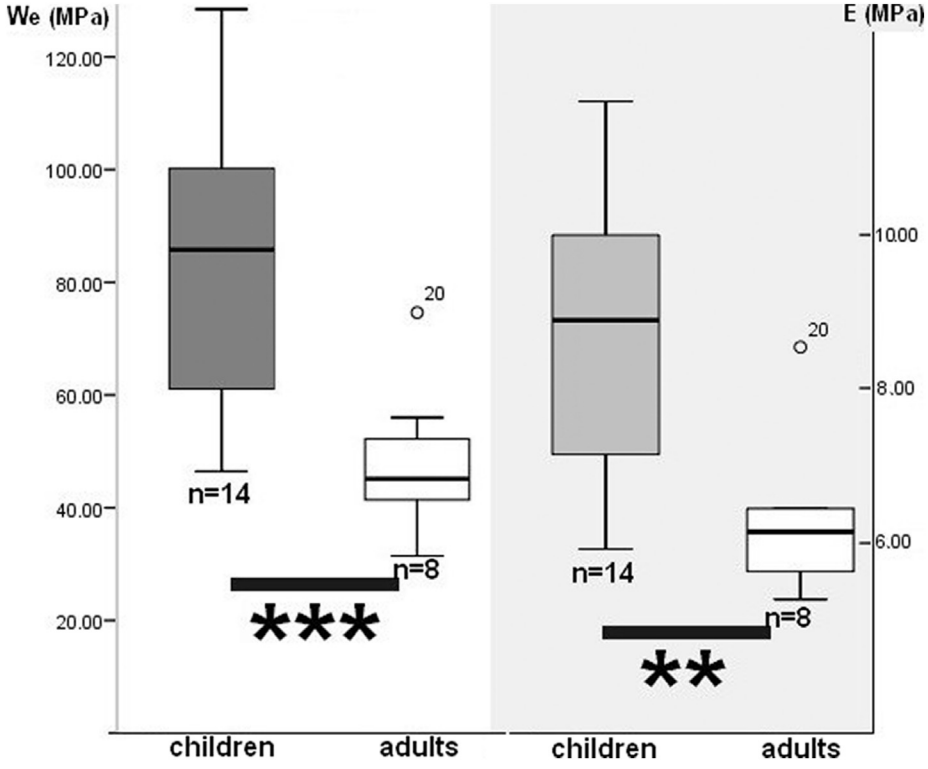


**Fig. 3.** Children's and adults' data combined (all collagen fibrils, n = 22) present a dissimilar monotonic relationship with age (Spearman's $\rho = -0.51$, $p = 0.015$).

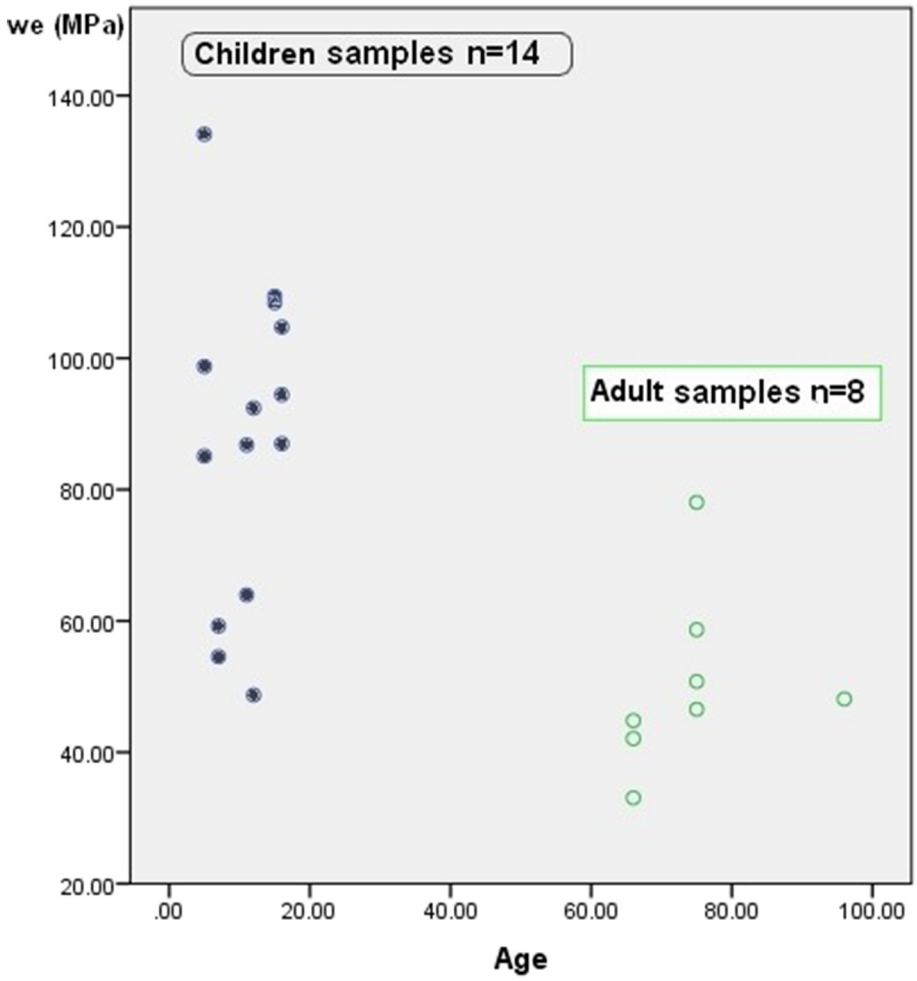


**Fig. 4.** Positive parametric linear relationship between numerical $W_e$ and covalent intermolecular connections for children's and adults' data separated (a), Positive parametric linear relationship between numerical $W_{tot}$ and covalent intermolecular connections only for adults' data (b), positive parametric linear relationship between numerical $E$ and covalent intermolecular connections only for children's data (c) and positive parametric linear relationship between numerical $W_e$ and cross-links ratio (CXLR) for children's and adults' data combined (d).

links in the children group indicates that the matrix of the bone samples studied is composed predominantly of newly synthesized collagen [74]. However, children's collagen contains more enzymatic collagen cross-links than adult collagen. More specifically, the number of intermolecular covalent liaisons is larger in the children population, suggesting that the high turnover in children's bone sample is associated with a high enzymatic collagen cross-links formation in order to maximize bone mechanical resistance. As a consequence, the results show a clear threshold value of the collagen cross-links ratio and intermolecular covalent liaisons between children and adults. Such structural difference could derive from a higher amount of posttranslational modification that favors enzymatic collagen cross-links formation in children's bone compared to adult's bone. Then, at the collagen fibrils level, the difference of mechanical behavior of the collagen fibrils between children and adults can be explained by both the density and maturity of enzymatic collagen cross-links. The higher intermolecular connectivity in children population allows the collagen fibrils to better sustain loads and then display a larger elastic response than the adult population. Indeed, a collagen fibril can be considered like a succession of springs connected both in series and in parallel. A minimum amount of cross-links

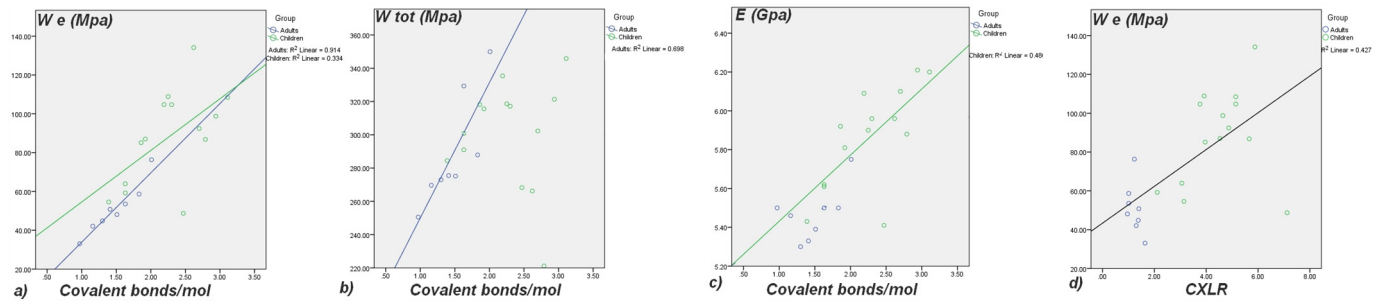


**Fig. 5.** Mean of collagen fibrils' *We* in children's group is significantly higher when compared to adults' group ($t$-test, $p = 0.001$); similarly, Modulus of Elasticity is significantly higher in children's group than in adults (Mann Whitney test, $p = 0.02$).

connected in series are necessary to insure the fibril integrity. Above this threshold, adding interconnected molecules by cross-linking is equivalent to adding springs in parallel and therefore increase the fibril's stiffness. This explains why children collagen, which contains more cross-links, displays an increased stiffness compared to adult collagen. However, divalent cross-links are not as strong as trivalent cross-links and favor intermolecular sliding and inhomogeneous deformation. This can explain the lack of link between cross-links density and plastic deformation in children collagen. In the adult population, due to the lower amount of intermolecular covalent liaisons, fewer molecules are interconnected explaining the lack of a clear correlation with elastic properties. However since trivalent cross-links form strong liaisons, they minimize intermolecular sliding by connecting multiple molecules in one point. Therefore collagen molecules are more likely to reach their plastic deformation regime simultaneously, explaining the direct relationship between cross-linking and toughness ($W_{tot}$) in adults [19]. This structural evolution of collagen could be a physiological response to the different environmental challenges that children and adults' skeletons are facing. In children, high turnover leads to an incomplete enzymatic collagen cross-links conversion allowing for higher toughness to account for larger risk of falls or injuries (green stick fractures) but at the expense of a higher energetic cost. Similarly, in adults, as the risk of falls and fractures drops, the cross-links conversion rate could increase in order to make bone stronger and to allow for more intense physical activities and to reduce the energetic cost associated with high turnover.

Although assessing differences in bone mechanical behavior between children and adults has been a major research question for the last decade [2,11–12,25,36,43,44,49,50,72], it is still difficult to define clear trends regarding bone mechanical properties evolution across ages. During growth, mineral and collagen phase mature simultaneously and this maturation process impacts bone mechanical behavior [29,20,59,60]. For instance, using samples extracted from the top part of the femur diaphysis (children aged from 4 to 15 and adults from 22 to 61), Öhman and colleagues have shown a lower *ex vivo* cortical strength and stiffness in children compared to adults and both depending on ash density [50]. Although mineral is likely to take part in the elastic deformation regime, the contribution of collagen to elasticity has been poorly described in the children population. Another study of Currey [17], using samples extracted from the mid-shaft of the femur (age range: 2 to 48 years old), observed that the bone specimens taken from children were weaker and less stiff (lower E) than those taken from adults, and also that they deflected more and absorbed more energy, without statistical evaluation. However in Currey's study, $W_e$ was not significantly different between children and elderly adults bone sample supporting our previous results about bone elasticity [9]. With these new results, we present a difference in the collagen fibril $W_e$ and E between the collagen phase of children and adult bone samples. This is in contradiction with the theoretical optimization hypothesis of stiffer bones in adults compared to children, which is currently used in computational models [34]. The development of newer approaches for modeling children bones is therefore necessary.

Like fibula, most of the bones have varying enzymatic collagen cross-links maturity ratios across ages [57,59,60]. Furthermore, children population presents a very low level of advanced glycation end products [11]. Then, our results establish for the first time a significant contribution of collagen maturity and density to collagen fibril elastic deformation of collagen across the lifetime (*i.e.* 5–96 year age range). Furthermore, our results show that $W_e$ is on average two times higher in children's than in adults' collagen fibril models (Fig. 4) and that 43% and 63% of the variance of $W_e$ can be explained by the variance of collagen cross-links ratio and intermolecular covalent liaisons, respectively. When we separated adults and children, our results show that the variance of $W_e$ remains explained by intermolecular covalent liaisons at 91% for adults and at 29% for children. However, collagen cross-links ratio does not explain the variance of $W_e$ neither for adults nor for children. Moreover, while 66% and 40% of the variance of $E$ is explained by intermolecular covalent liaisons and collagen cross-links ratio for all fibril tested, respectively; our results show that the variance of $E$ is solely explained in the children population sub group at 48% by intermolecular covalent liaisons. By comparing these new results to our previous work [11], our study shows that the number of intermolecular covalent liaisons created during the process of collagen fibril assembly is a key factor to understand the relationship between enzymatic cross-linking and mechanical properties. Furthermore, while collagen cross-links ratio is more and more investigated as a marker of bone mechanical behavior by using Raman or Infrared Spectroscopy [24,51,53], our results suggest that intermolecular covalent liaisons could be a better predictor regarding modulus of elasticity in children population and toughness in adult population.

Here, no significant direct relationship between the present collagen fibril scale mechanics and the macroscale mechanical properties measured in our previous study [11] have been depicted. However, we found a similar parametric linear correlation between $W_e$ and immature enzymatic collagen cross-links in the children population at both length scales. Among the possible reasons for the absence of direct relationship between the present collagen fibril scale mechanics and the macroscale mechanical properties measured in our previous study is that the loading modes studied are different (*i.e. in silico* tensile loading and *ex vivo* three-point bending). Also, while we focused on studying the collagen enzymatic collagen cross-links maturity in this study, other components of cortical bone, such as mineral content, hydration and hierarchical structure, are likely to play an important part in the mechanical behavior of the tissue and should be considered in future development [4,14,18,38,46,73]. Furthermore, because bone is known to be a hierarchical and heterogeneous material, a direct relationship between collagen fibril scale and macroscale properties is unlikely to be established [54]. Additionally, other numerical simulation studies on children's bone mechanical properties mainly focus on mineral content [34] and they also fail when results are compared to biomechanical tests [39–40,45]. Therefore, further analyses of the interplay between bone constituents are necessary. Finally, the bone samples we used cover a relatively short age range for both children and adults, and next efforts should concentrate in obtaining samples covering the gap region (16 to 66 years old).

The specificity of our collagen fibril molecular model is that the enzymatic collagen cross-links maturity and density can be modified to create fibrils with individual specific cross-linking compositions. In the molecular model, each collagen molecule presents two enzymatic collagen cross-links in its C-terminal and N-terminal domains, which follows the usual cross-linking pattern [23,33,47]. The stochastic nature of the fibrils creation algorithm implies that a 50% cross-linked fibril will have, on average, one cross-link created per collagen molecule [19]. However, enzymatic collagen cross-links distribution might not be equiproportional at each end of the collagen molecule which could affect the fibrillar mechanical response [33]. Furthermore, each molecular model has been built from experimental densities that represent an average value for the whole sample. Thus, since enzymatic collagen cross-links chemistry plays an important role in bone mechanics [47], introducing cross-link site-specificity and heterogeneities of density to the molecular model is necessary to investigate the multiscale link between mechanical properties.

Additionally, our results are for collagen fibrils that sustain loads in tension. In the daily life, loads in tension can be found in activities that involves hanging (pull-ups, climbing) or throwing (disk, hammer), and also in activities that involves long bones because they act as levers and are loaded in bending — combination of compression and tension — such as racket sports. As many orthopedics injuries in children are related to sport practice, our findings add to our global understanding of the mechanical properties of children bone and then might improve our strategy to prevent bone fracture in children population. The present results are also fundamental to understand the physiological and

pathological phenomena occurring in aging. By better understanding how bone is physiologically evolving with aging, development of better targeted therapies will be possible. While the link between enzymatic collagen cross-links and macroscopic mechanical behavior of bone tissue has been broadly established [3,8,16,19,35,48,57,59,60,67], there is still no means for pharmaceutical interventions to alter/repair enzymatic collagen cross-links for children with bone pathologies. Indeed, preventive and therapeutic strategies for skeletal diseases in children population rely on macroscopic mechanical properties standards that have been established from an interpolation of experimental newborn bone values to experimental adult bone values [34]. The thesis of this interpolation is an incremental increase of the stiffness marked by a stepwise mineralization of the collagen matrix. It is a direct relationship between bone mineral density and modulus of elasticity. However, the validity of this hypothesis shows limitations when pediatric numerical simulations are compared with biomechanical tests [39–40,45]. In our previous publication [11], we established a parametric link between immature enzymatic collagen cross-links and macroscopic *ex vivo* $W_e$ in the same children group. Here, our *in-silico* data goes beyond our experimental conclusion and show that 30% of the variance of collagen elastic energy is explained by the amount of immature enzymatic collagen cross-links and 62% by the amount of intermolecular covalent bonds formed by cross-linking, thus strongly suggesting that collagen maturation process is a major determinant of children's bone elasticity.

The higher amount of divalent cross-links in children compared to adult might be explained by a faster remodeling rate of children's bone tissue than in adults. Indeed, when the remodeling rate is high there is not enough time for enzymatic collagen cross-links to reach full maturity before the destruction of the bone occurs (*i.e.* higher amount of trivalent cross links than divalent). Then, children bone matrix is mainly composed of newly remodeled bone with a high amount of divalent cross-link compared to trivalent cross-links. While the high amount of divalent cross-links in children allow the bone to better sustain falls and not break, the remodeling rate of the bone is also dependent on the mechanical action on the osteocytes that activate osteoclasts – destruction of the bone matrix. Then, between the fact that children's bones have to sustain more falls and that activates the remodeling process, and children's bone have more divalent cross-links that allow them to sustain more falls by preventing the bone to break, it is not clear which of the two events should be considered the cause and which should be considered the effect. Whatever the explanation, we believe that enzymatic collagen cross-links could be enhanced using mechanical loading by means for musculoskeletal rehabilitation. Indeed, hyper- and micro-gravity, weight-bearing, and low intensity pulsed ultrasound (LIPUS) have shown distinct biological effects on bone collagen cross-links formation *in vitro* and *in vivo* (Mitsuru Saito and Marumo, 2010). Furthermore, we believe that diagnostic tools that detect collagen cross-links *ex vivo* – Raman spectroscopy [53] and biochemistry analysis ([32]; M Saito and Marumo, 2010) might be key to improve bone pathologies diagnosis in children.

## 5. Conclusion

We established a novel difference between children and adult bone in the elastic deformation of the collagen phase. Across lifetime and in parallel to the evolution of mineral density, our study indicates that the intermolecular connectivity in collagen induced by enzymatic cross-linking is a potential marker of elastic deformation in bone. This intermolecular connectivity is directly dependent on both enzymatic collagen cross-links maturity and density. Furthermore, and for the first time, we established a link between collagen fibril and macroscopic mechanical behavior by showing a similar parametric linear correlation between $W_e$ and immature collagen cross-links in the children population at two different bone scales. Our results extend the key role of collagen maturity in bone mechanical behavior to children's bone elasticity and then yield to new approaches for improving preventive and therapeutic strategies regarding skeletal diseases in children population.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2018.01.024.

## Compliance with ethical standards

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