



HAL
open science

Ratio between mature and immature enzymatic cross-links correlates with post-yield cortical bone behavior: An insight into greenstick fractures of the child fibula

Jean-Philippe Berteau, Evelyne Gineyts, Martine Pithioux, Cécile Baron, Georges Boivin, Philippe Lasaygues, Patrick Chabrand, Hélène Follet

► To cite this version:

Jean-Philippe Berteau, Evelyne Gineyts, Martine Pithioux, Cécile Baron, Georges Boivin, et al.. Ratio between mature and immature enzymatic cross-links correlates with post-yield cortical bone behavior: An insight into greenstick fractures of the child fibula. *BONE*, 2015, 79, pp.190-195. 10.1016/j.bone.2015.05.045 . hal-01296911

HAL Id: hal-01296911

<https://hal.science/hal-01296911>

Submitted on 27 Apr 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Ratio between mature and immature enzymatic cross-links impacts post-yield cortical bone behavior: an insight into *greenstick* fractures of the child fibula

BERTEAU Jean-Philippe PhD ¹⁻²⁻³, GINEYTS Evelyne PhD ⁴⁻⁵, PITHIOUX Martine PhD ¹,
BARON Cécile PhD ¹, BOIVIN Georges PhD ³⁻⁴, LASAYGUES Philippe PhD ², CHABRAND
Patrick Pr. ¹, FOLLET Hélène PhD ⁴⁻⁵

1 Institute of Movement Science (ISM) CNRS UMR 7287 Aix-Marseille University, av. de Luminy, F-13288 Marseille France,

2 Laboratory of Mechanics and Acoustics (LMA) CNRS UPR 7051 Aix -Marseille University Centrale Marseille, 31 chemin Joseph-Aiguier, F-13402 Marseille cedex 20 France

3 Department of Physical Therapy, College of Staten Island, City University of New York, NY 10314, United State of America

4 INSERM, UMR 1033, F-69008 Lyon, France

5 Université de Lyon, F-69008 Lyon, France

Corresponding author and address for reprint requests:

Jean-Philippe Berteau, Department of Physical Therapy, College of Staten Island, City University of New York, 2800 Victory Blvd, New York, NY 10314 United State of America

phone : (718) 982-2000

mail : jph.berateau@gmail.com

DISCLOSURE SECTION:

All authors state that they have no conflicts of interest.

1 ABSTRACT

2 As a determinant of skeletal fragility, the organic matrix is responsible for the post-yield and
3 creep behavior of bone and for its toughness, while the mineral apatite acts on stiffness.
4 Specific to the fibula and ulna in children, *greenstick* fractures show a plastic *in vivo*
5 mechanical behavior before bone fracture. During growth, the immature form of collagen
6 enzymatic cross-links gradually decreases, to be replaced by the mature form until
7 adolescence, subsequently remaining constant throughout adult life. However, the link
8 between the cortical bone organic matrix and *greenstick* fractures in children remains to be
9 explored. Here, we sought to determine: 1) whether plastic bending fractures can occur *in*
10 *vitro*, by testing cortical bone samples from children's fibula and 2) whether the post-yield
11 behavior (ω_p plastic energy) of cortical bone before fracture depends on the total quantity of
12 the collagen matrix, or on the quantity of mature and immature enzymatic cross-links and the
13 quantity of non-enzymatic cross-links. We used a two-step approach; first, a 3-point
14 microbending device tested 22 fibula bone samples from 7 children and 3 elderly adults until
15 fracture. Second, biochemical analysis by HPLC was performed on the sample fragments.
16 Results show a significant power correlation ($R^2= 0.70$) between the plastic energy dissipated
17 before fracture and the ratio of immature/mature cross-links. A collagen matrix with more
18 immature cross-links (i.e. a higher immature/mature cross-link ratio) is more likely to
19 plastically deform before fracture. We conclude that this ratio in the sub-nanostructure of the
20 organic matrix in cortical bone from the fibula may go some way towards explaining the
21 variance in post-yield behavior. From a clinical point of view, therefore, it partially explains
22 the presence of *greenstick* fractures in children.

23

24

25

1 KEY WORDS:

2 Children's Bone; Collagen Cross-Links; Post-Yield Behavior; Mechanical Properties.

3

1 Highlights

- 2 • A collagen matrix with more immature cross-links (i.e. higher immature/mature cross-
- 3 link ratio) is more likely to plastically deform before fracture.
- 4 • Cortical bone plastic energy variance is partially explained by the ratio between the
- 5 mature and the immature enzymatic cross-links.
- 6 • Pentosidine content is negatively related to plastic energy dissipated before cortical
- 7 bone fracture.
- 8

1 1. INTRODUCTION

2 Cortical bone is a highly specialized connective tissue composed of an organic matrix
3 of type I collagen with mineral hydroxyapatite and water. To reach its mature form, bone is
4 modeled by a process involving several steps. First, precursor cells turn into osteoblasts which
5 secrete collagen in a haphazard pattern called woven bone. Second, osteoclasts resorb the
6 woven bone and then osteoblasts form the lamellar cortical bone [1]. In this cortical bone,
7 type I collagen fibrils are assembled in a lamellar structure similar to plywood, where mineral
8 crystals (biological apatite) are deposited into the hole zones of collagen fibrils and between
9 collagen fibrils [2]. During modeling, a pediatric-specific orthopedic trauma called *greenstick*
10 fracture can occur on cortical bone. Clinically, *greenstick* fractures are *in vivo* plastic bending
11 fractures affecting the cortical part of children's long bones such as the fibula or the ulna [3];
12 they are bow fractures in which the bone becomes curved along its longitudinal axis. Often,
13 the fracture line does not propagate to the concave side of the bone, therefore showing
14 evidence of plastic deformation. The mechanical interpretation of these plastic bending
15 fractures is high toughness, meaning high plastic energy dissipated (ω_p) before fracture.
16 However, it is still unclear why children's bones foster this particular mechanical behavior.

17 The organic matrix is responsible for the post-yield and creep behavior of bone and for
18 its toughness, while the mineral apatite acts on stiffness [4,5]. In a previous study, our team
19 compared children's and elderly adults' acoustical velocities[6]; the dynamic modulus of
20 elasticity and Poisson's ratio were evaluated via an ultrasonic method and the static modulus
21 of elasticity was estimated from a 3-point microbending test. Neither elastic properties nor
22 density were statistically different between cortical bone samples from children and elderly
23 adults. Since numerous studies [5,7–11] show that human cortical bone elastic parameters are
24 linked to density, mineral content, porosity and more generally to its mineral component, we

1 therefore decided here to examine how cross-links affect the post-yield behavior of cortical
2 bone samples and their collagen matrix structure.

3 In human bone, type I collagen represents 90% of the organic phase, with collagen
4 molecules consisting of three polypeptide strands. Stabilization of newly-formed collagen
5 fibers is initially achieved by the formation of covalent cross-links between neighboring
6 collagen molecules. The cross-links are formed via two different pathways. One pathway
7 involves the oxidative deamination of the ϵ -amino group on lysyl or hydroxylysyl side chains
8 of telopeptides, resulting in the formation of two aldehydes, allysine and hydroxyallysine and
9 controlled by the enzyme lysyl-oxidase. It leads to the formation of divalent cross-links which
10 stabilize the immature collagen fibers, such as dihydroxylysinonorleucine (DHLNL) and
11 hydroxylysinonorleucine (HLNL). These then react with another telopeptide aldehyde group
12 to form mature trivalent pyridinium cross-links, such as pyridinoline (PYD) and
13 deoxypyridinoline (DPD) and pyrrole, which stabilize the collagen fibrils with age. The other
14 pathway operates at a higher level, via the non-enzymatic glycation mechanism which forms
15 advanced glycation products (AGEs) such as pentosidine (PEN) following tissue maturation.
16 Some *in vitro* and *in vivo* studies suggest that PEN may influence bone fracture [12] [13];
17 however the impact of non-enzymatic cross-links is still unclear.

18 We chose to study the lower extremity of a long bone, the fibula, where plastic
19 bending fractures typically occur. Our objective was to determine: 1) whether plastic bending
20 fractures occur when samples are tested *in vitro*; 2) whether the mechanical properties of
21 cortical bone depend on the quantity of the collagen matrix or on the quantity of mature or
22 immature enzymatic or non-enzymatic cross-links. We used a two-step method: first,
23 mechanical testing until fracture with a 3-point microbending device and second, biochemical
24 analysis by high performance liquid chromatography (HPLC) on 1g of the bone samples
25 reduced to fragments.

1 2. MATERIALS AND METHODS

2 2.1. Bone samples

3 Child cortical bone samples from auto transplants were obtained from children of
4 Western European descent requiring surgery (Timone Hospital, Marseille, France) and all (or
5 their legal guardians) gave informed written consent in accordance with the French Code of
6 Public Health (Code de la Santé Publique Français) and approved by the Ethics Committee
7 for the Protection of Persons. All the auto-transplants were excised from a non-pathological
8 area at the bottom of the fibula, 5 cm above the ankle and unused fragments (waste) were
9 kept. Adult bone samples from elderly adult donors were extracted from the same location
10 (INSERM UMR 1033 and IFSTTAR UMR-T 9406, University of Lyon, France) and all
11 cortical bone samples were cut using a low-speed diamond saw (Isomet 1000, Buehler, Lake
12 Bluff, IL, USA) to obtain parallelepiped samples (plane and parallel surfaces). A total of 22
13 cortical bone samples from 10 subjects (min. 5 years, max. 99 years) were studied, 14 from 7
14 children (mean age 10.14 ± 4.56 years old) and 8 from 3 elderly adults (mean age 79 ± 15.39
15 years old). All samples were measured with a digital caliper (Absolute digimatik solar,
16 Mitutoyo, Kanagawa, Japan, measurement error of 0.03 mm.) and were weighed on a
17 micrometer weighing scale with a density kit (Voyager 610 GX, Ohaus Corporation, Florham
18 Park, NJ, USA, measurement uncertainty of 0.001 g/cm^3) [6]. Specimens were vacuum
19 packed and stored at -20°C in a tissue with phosphate buffered saline solution.

20 2.2. Mechanical measurements

21 A 3-point microbending testing system appropriate for such small samples was
22 designed and mounted on a Universal Testing Machine (Instron 5566A, Norwood, MA,
23 USA). To test our cortical bone samples, the 3-point microbending testing system was
24 customized to adapt its dimensions to the sample dimensions and to respect a span-to-depth
25 ratio of 8:1 and an average width-to-thickness ratio of about 4, which corresponds to a shear

1 factor of 0.833 [14]. A pre-force of 5 N was applied on the sample, then the test started with a
2 displacement speed of 0.1 mm/min (close to static testing conditions) until fracture. The test
3 provided a force/displacement curve for each sample, which we transformed into a
4 strain/stress curve by dividing force and displacement by cross-sectional area and length to
5 deduce the elastic and plastic properties (Figure 1). The modulus of elasticity was previously
6 calculated [6]. Toughness was assessed by calculating the integration of area under
7 stress/strain curve leading to mechanical energies dissipated before fracture: ω_e , ω_p and ω_{tot}
8 for elastic, plastic and total energies respectively. The yield point was the intersection
9 between the power law curve, fitted to the stress–strain data using a 0.2% offset as the cutoff
10 for linearity in the elastic domain, and the stress/strain curve. The measurement error for the
11 cell force was estimated at 0.23%.

12 2.3. Biochemical measurements

13 To quantify the major cross-links found in type I collagen, after the 3-point microbending
14 test, any sample weighing more than 1g was subjected to a complete biochemical analysis.
15 These samples were ground in liquid nitrogen, decalcified in 0.5M EDTA solution for 96
16 hours and reduced with NaBH₄ to stabilize the labile divalent immature cross-links and to
17 form acid-resistant dihydroxylysinonorleucine (DHLNL) and hydroxylysinonorleucine
18 (HLNL) before acid hydrolyzing with 6M hydrochloric acid. The cross-links were extracted
19 from the acid hydrolysate using a solid phase extraction column (Chromabond cross-links®,
20 Macherey Nagel, Düren, Germany). Then, they were separated on a C18 Atlantis® T3
21 reversed-phase column, with heptafluorobutyric acid as volatile ion-pairing reagent in an
22 acetonitrile-water mobile phase on an HPLC system equipped with an Alliance 2695
23 separation module, a Waters Micromass® ZQ™ Single Quadrupole Mass Spectrometer, a
24 2647 Multi 1 fluorescence detector and Empower2 chromatography data software (Waters
25 Corp. Milford, MA, USA). The detection of DHLNL, HLNL, PYD and DPD was performed

1 by electrospray ionization mass spectrometry in a positive ion mode with selected ion
2 recording [15]. The ratio of (DHLNL+HLNL)/ (PYD+DPD) represents the state of cross-link
3 maturation and thus the maturation of the collagen matrix. Pentosidine (PEN) was quantified
4 by fluorescence at an emission of 385 nm and an excitation of 334 nm [16], the evaluation of
5 this AGE representing the glycation state of the collagen matrix. Then, the proportion of bone
6 collagen was analyzed by measuring hydroxyproline (OHP); unfortunately, however, the
7 quantity of cortical bone samples for three children and one adult was insufficient to allow
8 measurement (missing data (MD) in table 1).

9 2.4. Statistical analysis

10 22 samples were tested and values for multiple samples from the same donor were
11 averaged, yielding one value per donor (n=10). Normal distribution was tested with the
12 Shapiro-Wilk test, Pearson correlation was performed in the event of normal distribution and
13 Spearman correlation in the event of non-normal distribution. In addition, indicative
14 comparisons were performed between the children (n=7) and the adults (n=3). All statistical
15 tests were performed with SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for
16 Windows, Version 22.0. Armonk, NY: IBM Corp).

17

18 3. RESULTS

19 3.1. Mechanical measurements

20 All the stress/strain curves from the microbending test are shown in Figure 2 and
21 toughness data (elastic, plastic and total energies) are listed per sample and per subject (mean)
22 in table 1. The ω_e , ω_p and ω_{tot} were respectively 2.8, 4.7 and 4.4 times higher in children than
23 in elderly adults. The ω_p was lower and more clustered in adults compared to children (values
24 more dispersed and higher); a non-parametric negative significant correlation was found
25 between ω_{tot} and Age (Spearman: -0.70, p=0.02; plotted on Figure3).

3.2. Biochemical measurements

The experimental results of the biochemical evaluation of DHLNL, HLNL, PYD, DPD, PEN, ratio of (DHLNL+HLNL)/ (PYD+DPD) and % of collagen are listed per sample and per subject (mean) in Table 1. The proportion of collagen does not vary across age groups (roughly 22 %). PEN content and PYD+DPD content were higher in the elderly adults than in the children (7 and 1.3 times, respectively). Conversely, the concentration of DHLNL+HLNL and the ratio of (DHLNL+HLNL) / (PYD+DPD) were higher in the children than in the elderly adults (3.1 and 3.8 times, respectively). A non-significant correlation but a negative trend between ratio of (DHLNL+HLNL)/ (PYD+DPD) and age was observed (Spearman: -0.62 p=0.053).

3.3. Dependence between mechanical and biological parameters

Values of (DHLNL+HLNL), PEN, % of collagen, ratio of (DHLNL+HLNL)/ (PYD+DPD), ω_e , ω_p and ω_{tot} were normally distributed; values of (PYD+DPD) and age (in years) were non-normally distributed. Parametric positive significant correlation was found both between ω_{tot} and ratio of (DHLNL+HLNL)/ (PYD+DPD) (Pearson: 0.66, p=0.04) and between ω_p and ratio of (DHLNL+HLNL)/ (PYD+DPD) (Pearson: 0.71, p=0.02, with a power correlation: $\omega_p = 0.81$ (ratio (DHLNL+HLNL) / (PYD+DPD) ^{1.2}, R² = 0.77 plotted on Figure 4a). Parametric negative significant correlation was found both between ω_{tot} and PEN (Pearson: -0.77, p=0.008) and between ω_p and PEN (Pearson: -0.80, p=0.005 and with a negative logarithm correlation: $\omega_p = -1.99 \ln (\text{PEN}) + 3.98$ plotted on Figure 4b).

4. DISCUSSION

The main goal of the present study was to determine *in vitro* whether plastic bending fractures occur on samples of child bone and whether the post-yield behavior of cortical bone depends on the quantity or the quality of its collagen content, or on the quantity of enzymatic

1 (DHLNL, HLNL, PYD, DPD) and non-enzymatic cross-links (PEN) in the collagen matrix,
2 or on the ratio of immature/ mature ((DHLNL+HLNL) / (PYD+DPD)) enzymatic cross-links.

3 To the authors' knowledge, the mechanical behavior of physiological child bone has
4 been quantitatively investigated by very few *in vitro* mechanical studies, all using destructive
5 tests on dry samples [17,18]. One [17], using samples extracted from the mid-shaft of the
6 femur (age range: 2 to 48 years old), observed that the bone specimens taken from children
7 were weaker and less stiff than those taken from adults, and also that they deflected more and
8 absorbed more energy, without statistical evaluation. This is in agreement with clinical
9 observations showing that young bone absorbs more energy before breaking, which can lead
10 to plastic deformation in *greenstick* form. The load-deformation curves yielded by our
11 mechanical tests also show that there is greater energy absorption by the young bone, no
12 doubt as a result of the greater ability of such bone to undergo plastic as opposed to elastic
13 deformation[19]. Thus far, to our knowledge, no attempt has been made to explore the
14 biological reasons for the plastic bending of human cortical bone. Another study [18], using
15 samples extracted from the top part of the femur diaphysis (children aged from 4 to 15 and
16 adults from 22 to 61), showed a difference in cortical strength and stiffness depending on ash
17 density, although the compressive yield strain was the same. However, no parameters were
18 related to post-yield behavior.

19 The present study, using fibula samples (children aged from 5 to 16 and adults from 66
20 to 99), mechanically evaluated post-yield behavior and its organic matrix via HPLC analysis.
21 When the mechanical measurements and the biochemical measurements are compared, it
22 appears that both plastic strain energy (ω_p) and ratio of (DHLNL + HLNL)/ (PYD + DPD) are
23 influenced by the ageing process and are significantly correlated ($R^2 = 0.70$), meaning that
24 70% of the variance in bone-tissue ω_p is explained by the ratio of enzymatic to non-
25 enzymatic cross-links alone. The ratio of (DHLNL + HLNL)/ (PYD + DPD) represents the

1 state of cross-link maturation, and the relationship we found here between this ratio and ω_p
2 shows that a collagen with more immature cross-links (i.e. a higher immature/mature cross-
3 link ratio) is more likely to plastically deform before fracture. Moreover, pentosidine (PEN)
4 content is negatively correlated to ω_p before cortical bone fracture explaining 89% of its
5 variance in bone-tissue, which shows that the presence of PEN reduces the bone's ability to
6 plastically deform. A correlation between two variables originally assessed at different levels
7 might lead to underestimation of this correlation, and a major contribution here consists in the
8 values being separated into two groups: the children, with ω_p above 2 MPa and with a ratio
9 below 2, and the adults, with ω_p below 2 MPa and with a ratio above 2. Our results suggest
10 that cortical bone samples with a ratio of $(\text{DHLNL} + \text{HLNL}) / (\text{PYD} + \text{DPD})$ above 2 have a
11 greater capacity for plastic deformation (*greenstick* fractures) and that conversely, cortical
12 bone samples with a ratio lower than 2 are unable to plastically deform (brittle fractures).

13 For all the cross-links of collagen analyzed (PEN, DHLNL + HLNL, PYD + DPD), as
14 well as for the ratio of $(\text{DHLNL} + \text{HLNL}) / (\text{PYD} + \text{DPD})$, our results are in agreement with
15 Saito and al. [20]. Enzymatic cross-links (DHLNL+HLNL) gradually decrease from
16 childhood to adolescence, to be replaced in part by (PYD+DPD); our results on both children
17 and adults for human fibula bone are consistent with the literature [20]. This may indicate that
18 the structure of collagen becomes increasingly hierarchized with increasing maturity of
19 collagen, reflecting the replacement of *woven* bone (children) by *Haversian lamellar* bone
20 (adults). Being non-enzymatic, PEN cross-links reflect relative tissue age and matrix turnover
21 rate. The low quantity of PEN observed in early years may be due to the rapid increase in
22 collagen content from the kinetics of bone modeling that is not subject to modification.
23 However, we did not quantify the reducible cross-links which may contribute to skeletal
24 fragility and we only analyzed PEN, which explained 40% of the variance in bulk fluorescent
25 AGE, only a portion of the total fluorescent AGEs that accumulate with tissue age in bone

1 [21]. This is supported by findings on animals (rabbits and mice) as shown by Isaksson [22].
2 However, since differences in PEN content have been found between adult and elderly bones
3 [13,19], inclusion of more individuals and possibly middle-aged (30-50 years old) adult bones
4 would strengthen our results. Nevertheless, these findings based on 14 samples consider the
5 growing process and are the first to show the ratio of $(DHLNL + HLNL) / (PYD + DPD)$ and
6 the dissipated mechanical energies, analyzed and correlated on the same samples. Similar
7 collagen content results are obtained here for children and adults, while Saito and Marumo
8 [23] reported an increase in collagen content during growth (new-born infants to 20-year-
9 olds) for bones from many different locations in the body. These authors studied only one
10 long bone from the lower extremity (middle of the diaphysis of the tibia) and their results
11 ranged from 20% to 22% collagen in bone incised during growth. This is similar to our
12 findings, showing both the importance of the location of the bone sample and the potential
13 effect of mechanical stress acting on the whole bone. Saito et Muramo [23] reported between
14 15% and 17% collagen in the bone incised from adults up to 60 years old, which differs from
15 our results (roughly 22%) for elderly adults.

16 Although all samples were prepared identically, one limitation in the preparation
17 process should be pointed out: the children's samples were taken *in vivo*, whereas the elderly
18 adults' samples were obtained from cadavers. The data in this report is difficult to correlate,
19 as gender is not a component of the analyses; it is hard to address this component because of
20 the difficulty of obtaining bone samples. It should be noted that the cross-links studied here
21 are merely some of the numerous collagen cross-links in bone tissue. The main issue is the
22 freezing period, which is difficult to establish when bone is from multiple sources. But frozen
23 bone can safely be used for mechanical testing, at least for storage periods of up to one year
24 [24] (the case here), during which it can be assumed that no major changes occur in the
25 molecular structure of the collagen. However, there are limitations to our mechanical tests due

1 to the size of the samples. First, Spatz et al. [25] established that the minimum span-to-depth
2 ratio in 3-point microbending should be around 20:1 (preferably 25:1) to estimate the
3 longitudinal modulus of elasticity of bone material and, consequently, the mechanical
4 energies dissipated before fracture. The ratio applied in the present study (8:1) is imposed by
5 the very small dimensions of the bone and of the surgical waste we used. Second, we
6 characterized the longitudinal axis of the samples; elderly cortical and trabecular bone are
7 both orthotropic [2], so it might be assumed that child cortical bone is also an anisotropic
8 tissue. A study might therefore be expected to test a transverse direction (radial or
9 circumferential) so as to conclude on the total dissimilarity of bending behavior in children's
10 and adults' samples. However, the fracture mechanism of "*greenstick* fractures" does not act
11 in a transversal direction. To investigate the clinical differences between bone from children
12 and from elderly adults, we therefore tested the bone samples in the longitudinal direction
13 only, because mimicking clinical fracture in our mechanical tests appeared to us the best way
14 to reveal true bending behavior. Moreover, since fracture in human cortical bone was found
15 by Spatz et al. [25] to be consistent with strain-controlled failure, and the influence of
16 microstructure can be described in terms of several toughening mechanisms, we tested all our
17 samples in quasi-static conditions in order to be able to compare data for growing and for
18 mature bones.

19 The toughness of a material is determined not only by its composition, but also by the
20 ability of its microstructure to dissipate deformation energy without propagation of the crack
21 [26]. Mature bone is a brittle micro-cracking material that derives its fracture resistance
22 (toughness) from its ability to form microcracks that absorb energy and delay the propagation
23 of a major crack [26][27]. Nalla et al. [28] compared young adult and old adult cortical bone
24 samples and found that these latter may have reduced mechanical properties due to the
25 presence of more microcracks, older bone being more susceptible to developing microcracks

1 at a given strain level. Since broken inter-fibrillar cross-links help dissipate energy, it is
2 tempting to speculate from our results that the non-mature cross-links broke later than the
3 mature cross-links, as has recently been suggested by numerical simulations [29].

4 From a fracture-resistance point of view, the hierarchized organization of the tissue
5 according to the plywood model and the associated variation in fibril angles across bone
6 tissue may lead to a more ductile, and thus fracture-inducing, behavior of bone tissue [30].
7 From this point of view, it would be interesting to compare different mechanical actions
8 impacting different fibril angles in conjunction with a cross-link study [31]. Furthermore,
9 although an improved understanding of static mechanical properties is very useful,
10 considering that the majority of bone fractures occur under dynamic conditions such as
11 accidents or sporting activities for the young and falls for the elderly, and in view of the
12 dynamic nature of physiotherapy [32], it would seem desirable to explore the dynamic
13 mechanical properties of children's bone. Although it was not investigated in the present
14 study, hydration could be of importance. We do not yet know the exact mechanism of
15 hydration within an osteon, but this should not prevent us from addressing the question of the
16 effect of pore water on bone viscoelasticity. Interactions among collagen, moisture, and
17 minerals may be key to bone viscoelasticity, and future work could usefully investigate this,
18 using a different kind of mechanical test, such as the Fatigue test. It has also been shown that
19 the brittle failure of various hydroxyapatite biomaterials characterized by different porosities
20 could be explained by the failure characteristics of individual crystals and by the
21 microstructure these crystals build up [33]. Consequently, assessing the effects of water and
22 of crystal organization constitute valuable future research goals.

23

24 5. CONCLUSION

1 The aim of this study was to assess both the post-yield mechanical behavior of child
2 and elderly adult cortical bone and the characteristics of the collagen matrix. We find a link
3 between the plastic behavior and the sub-nanostructural organization of the organic matrix in
4 cortical bone. Under our experimental conditions, a proportion of the variance in cortical bone
5 plastic energy was explained by the ratio between the mature and the immature enzymatic
6 cross-links, suggesting that these variables are one determinant of the plastic properties of
7 cortical bone tissue. In conclusion, our findings indicate that the ratio of (DHLNL+HLNL) /
8 (PYD+DPD) at the sub-nanostructural level of the organic matrix increases with age and
9 impacts the macroscopic mechanical behavior of cortical bone.

11 AKNOWLEDGEMENTS

12 This study was based on research supported by the French National Research Agency
13 (BioGMID Program ANR under Grant n°183692 and MALICE Program ANR under Grant
14 n°BS09-032). We thank the Timone Hospital surgery team and the donors or their legal
15 guardians. The authors are gratefully thankful to Dr. Frédéric Rongieras (Hôpital Desgenettes,
16 Lyon) for the dissection of adult bones. Our thanks also to Marjorie Sweetko for English
17 language revision.

20 REFERENCES

- 21 [1] Marks SC, Popoff SN. Bone cell biology: the regulation of development, structure,
22 and function in the skeleton. *American Journal of Anatomy* 2005;183:1–44.
- 23 [2] Rho JY, Kuhn-Spearing L, Zioupos P. Mechanical properties and the hierarchical
24 structure of bone. *Medical Engineering & Physics* 1998;20:92–102.

- 1 [3] Salter R, Harris R. Injuries Involving the Epiphyseal Plate. *J Bone Joint Surg Am*
2 1963;45:587–622.
- 3 [4] John D. Currey. Role of collagen and other organics in the mechanical properties of
4 bone. *Osteoporos Int* 2003;14 Suppl 5:S29–36. doi:10.1007/s00198-003-1470-8.
- 5 [5] Bala Y, Depalle B, Douillard T, Meille S, Clément P, Follet H, et al. Respective roles
6 of organic and mineral components of human cortical bone matrix in micromechanical
7 behavior: An instrumented indentation study. *Journal of the Mechanical Behavior of*
8 *Biomedical Materials* 2011;4:1473–82. doi:10.1016/j.jmbbm.2011.05.017.
- 9 [6] Berteau J-P, Baron C, Pithioux M, Launay F, Chabrand P, Lasaygues P. In vitro
10 ultrasonic and mechanic characterization of the modulus of elasticity of children cortical
11 bone. *Ultrasonics* 2013. doi:10.1016/j.ultras.2013.09.014.
- 12 [7] McCalden RW, McGeough JA, Barker MB, Court-Brown CM. Age-related changes
13 in the tensile properties of cortical bone. The relative importance of changes in porosity,
14 mineralization, and microstructure. *J Bone Joint Surg Am* 1993;75:1193–205.
- 15 [8] Zhu M, Keller TS, Spengler DM. Effects of specimen load-bearing and free surface
16 layers on the compressive mechanical properties of cellular materials. *Journal of*
17 *Biomechanics* 1994;27:57–66. doi:10.1016/0021-9290(94)90032-9.
- 18 [9] Keller TS. Predicting the compressive mechanical behavior of bone. *Journal of*
19 *Biomechanics* 1994;27:1159–68. doi:10.1016/0021-9290(94)90056-6.
- 20 [10] Burr DR. *The mechanical adaptations of bones*. By John Currey. Princeton, New
21 Jersey: Princeton University Press. 1984. ix + 294 pp., figures, tables, references, index.
22 \$37.50. *American Journal of Physical Anthropology* 1985;68:141–2.
23 doi:10.1002/ajpa.1330680117.
- 24 [11] Currey JD, Butler G. The mechanical properties of bone tissue in children. *J Bone*
25 *Joint Surg Am* 1975;57:810–4.

- 1 [12] SY T, Vashishth D. The relative contributions of non-enzymatic glycation and
2 cortical porosity on the fracture toughness of aging bone. *Journal of Biomechanics*
3 2011;44:330–6.
- 4 [13] Wang X, Shen X, Li X, Mauli Agrawal C. Age-related changes in the collagen
5 network and toughness of bone. *Bone* 2002;31:1–7.
- 6 [14] Brancheriau L. Influence of cross section dimensions on Timoshenko's shear factor –
7 Application to wooden beams in free-free flexural vibration. *Annals of Forest Science*
8 2006;63:319–21. doi:10.1051/forest:2006011.
- 9 [15] Gineyts E, Borel O, Chapurlat R, Garnero P. Quantification of immature and mature
10 collagen crosslinks by liquid chromatography–electrospray ionization mass spectrometry in
11 connective tissues. *Journal of Chromatography B* 2010;878:1449–54.
12 doi:10.1016/j.jchromb.2010.03.039.
- 13 [16] Viguet-Carrin S, Gineyts E, Bertholon C, Delmas PD. Simple and sensitive method
14 for quantification of fluorescent enzymatic mature and senescent crosslinks of collagen in
15 bone hydrolysate using single-column high performance liquid chromatography. *Journal of*
16 *Chromatography B* 2009;877:1–7. doi:10.1016/j.jchromb.2008.10.043.
- 17 [17] Currey JD. Changes in the impact energy absorption of bone with age. *Journal of*
18 *Biomechanics* 1979;12:459–69.
- 19 [18] Öhman C, Baleani M, Pani C, Taddei F, Alberghini M, Viceconti M, et al.
20 Compressive behaviour of child and adult cortical bone. *Bone* 2011;49:769–76.
- 21 [19] Viguet-Carrin S, Roux JP, Arlot ME, Merabet Z, Leeming D., Byrjalsen I, et al.
22 Contribution of the advanced glycation end product pentosidine and of maturation of type I
23 collagen to compressive biomechanical properties of human lumbar vertebrae. *Bone*
24 2006;39:1073–9. doi:10.1016/j.bone.2006.05.013.

- 1 [20] Saito M, Fujii K, Marumo K. Degree of mineralization-related collagen crosslinking
2 in the femoral neck cancellous bone in cases of hip fracture and controls. *Calcified Tissue*
3 *International* 2006;79:160–8.
- 4 [21] Karim L, Vashishth D. Heterogeneous Glycation of Cancellous Bone and Its
5 Association with Bone Quality and Fragility. *PLoS ONE* 2012;7:e35047.
6 doi:10.1371/journal.pone.0035047.
- 7 [22] Isaksson H, Harjula T, Koistinen A, Iivarinen J, Seppänen K, Arokoski J, et al.
8 Collagen and mineral deposition in rabbit cortical bone during maturation and growth: effects
9 on tissue properties. *Journal of Orthopaedic Research* 2010;28:1626–33.
- 10 [23] Saito M, Marumo K. Collagen cross-links as a determinant of bone quality: a possible
11 explanation for bone fragility in aging, osteoporosis, and diabetes mellitus. *Osteoporosis*
12 *International* 2010;21:195–214.
- 13 [24] Van Haaren EH, van der Zwaard BC, van der Veen AJ, Heyligers IC, Wuisman PI,
14 Smit TH. Effect of long-term preservation on the mechanical properties of cortical bone in
15 goats. *Acta Orthopaedica* 2008;79:708–16. doi:10.1080/17453670810016759.
- 16 [25] Spatz H-C, O’Leary EJ, Vincent JFV. Young’s Moduli and Shear Moduli in Cortical
17 Bone. *Proceedings of the Royal Society B: Biological Sciences* 1996;263:287–94.
18 doi:10.1098/rspb.1996.0044.
- 19 [26] Zioupos P, Hansen U, Currey JD. Microcracking damage and the fracture process in
20 relation to strain rate in human cortical bone tensile failure. *Journal of Biomechanics*
21 2008;41:2932–9.
- 22 [27] Follet H, Farlay D, Bala Y, Viguet-Carrin S, Gineyts E, Burt-Pichat B, et al.
23 Determinants of Microdamage in Elderly Human Vertebral Trabecular Bone. *PLoS ONE*
24 2013;8:e55232. doi:10.1371/journal.pone.0055232.

- 1 [28] Nalla RK, Kinney JH, Ritchie RO. Mechanistic fracture criteria for the failure of
2 human cortical bone. *Elastic* 2003;11:2–0.
- 3 [29] Depalle B, Qin Z, Shefelbine SJ, Buehler MJ. Influence of cross-link structure,
4 density and mechanical properties in the mesoscale deformation mechanisms of collagen
5 fibrils. *Journal of the Mechanical Behavior of Biomedical Materials* 2014.
6 doi:10.1016/j.jmbbm.2014.07.008.
- 7 [30] Peterlik H, Roschger P, Klaushofer K, Fratzl P. From brittle to ductile fracture of
8 bone. *Nature Materials* 2005;5:52–5.
- 9 [31] Siegmund T, Allen MR, Burr DB. Failure of mineralized collagen fibrils: Modeling
10 the role of collagen cross-linking. *Journal of Biomechanics* 2008;41:1427–35.
- 11 [32] Berteau J-P. La biomécanique de l’os de l’enfant en croissance, une aide à la prise en
12 charge kinésithérapique. *Kinésithérapie, La Revue* 2013;13:16–21.
13 doi:10.1016/j.kine.2013.06.014.
- 14 [33] Fritsch A, Hellmich C, Dormieux L. Ductile sliding between mineral crystals
15 followed by rupture of collagen crosslinks: Experimentally supported micromechanical
16 explanation of bone strength. *Journal of Theoretical Biology* 2009;260:230–52.
17 doi:10.1016/j.jtbi.2009.05.021.

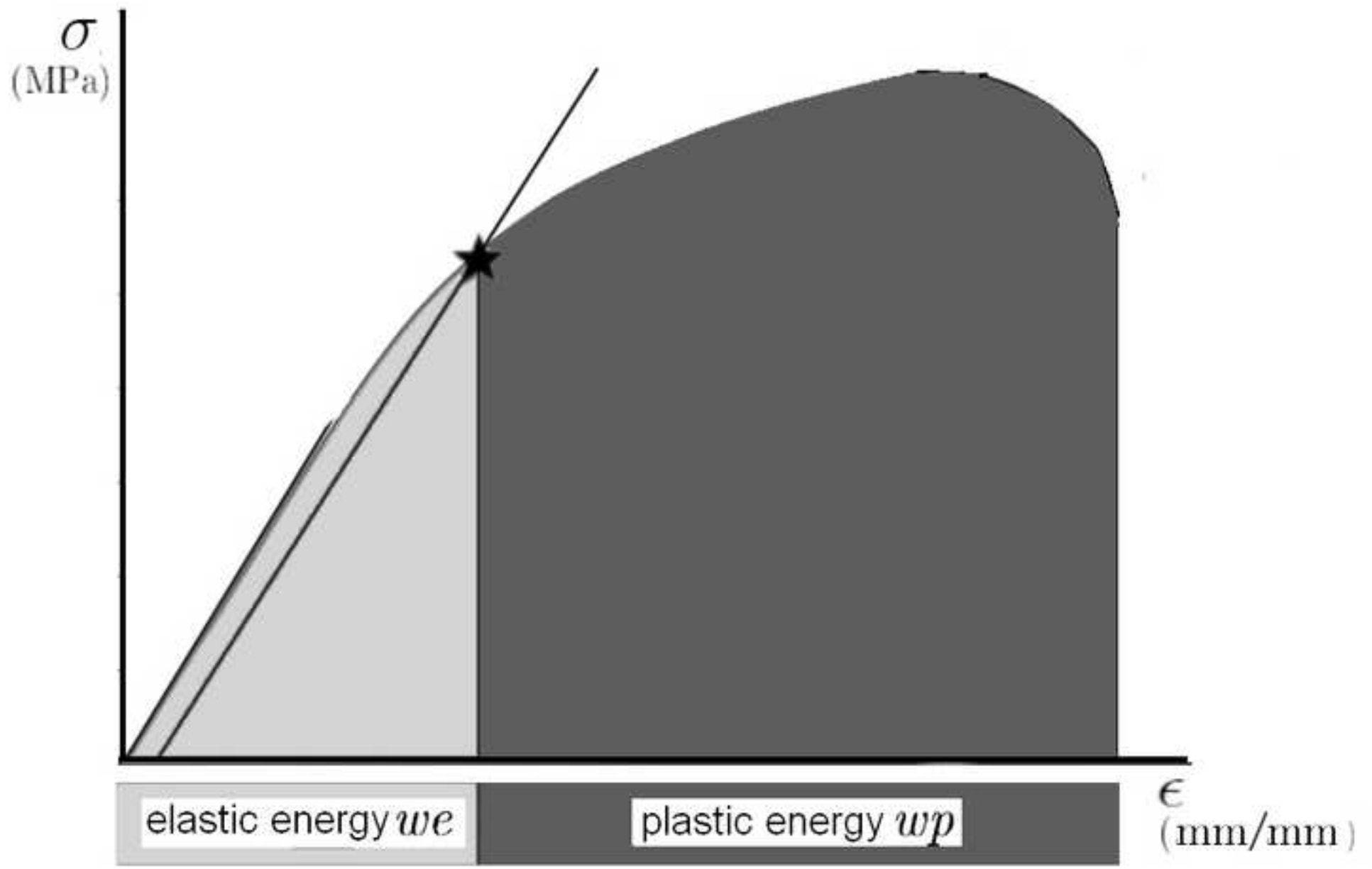
18
19
20
21
22
23
24
25

- 1 Figure Legends
- 2 Figure 1: Microbending test curve (strain/stress) and calculation of energies
- 3 Figure 2: Microbending test curves (strain/stress) for all children's and elderly adults' cortical
- 4 bone samples. Graphs are separate into age, for each range of age. For example, upper left
- 5 graph represents 3 tests: child number1 has one sample, and children number two has two
- 6 samples (indicated in the legend, by number 2 & 3).
- 7 Figure 3: ω_{tot} as a function of age for elderly adults, for children.
- 8 Figure 4: Dependence between ω_p and ratio of (DHLNL + HLNL)/ (PYD + DPD) (a) and
- 9 PEN (b) for elderly adults' group values (white dots) and children's group values (black dots)
- 10
- 11 Table legends
- 12 Table 1: Age, sex, dimensions, toughness and biochemical measurements of all samples.
- 13 Mean of toughness and biochemical measurements for each elderly adult and each child (MD
- 14 means missing data).

Table(s)

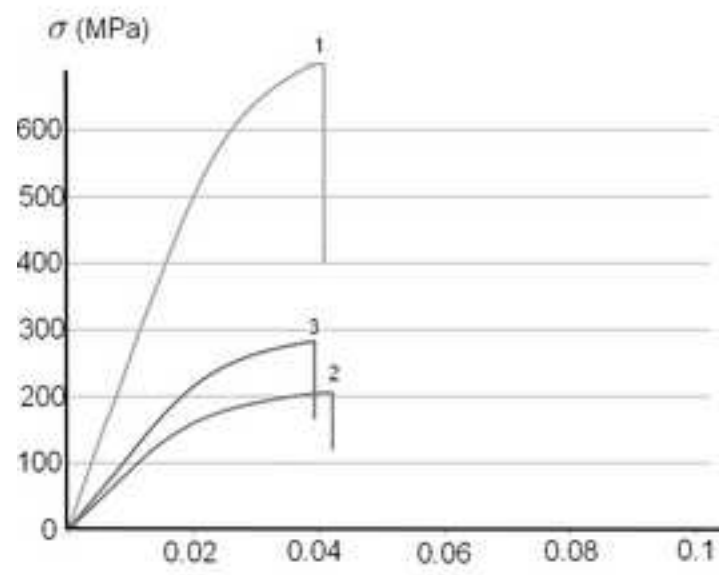
Category	Age (years)	Samples	ω elastic (GPa)	ω plastic (GPa)	ω total (GPa)	DHLNL+HLNL (mmol /mol Coll)	PYD+DPD (mmol /mol Coll)	ratio (DHLNL+HLNL) / (PYD+DPD)	PEN (mmol /mol Coll)	% collagen
Child 1	5.0	1	0.05	2.64	2.69	2049.139	354.304	5.784	0.392	MD
		2	0.25	3.25	3.50	2274.243	487.584	4.664	0.444	25.2
mean			0.15	2.95	3.10	2161.691	420.944	5.224	0.418	25.2
standard deviation			0.14	0.43	0.57	159.173	94.243	0.791	0.037	
Child 2	5.0	3	0.59	6.59	7.18	1261.934	319.747	3.947	1.159	21.8
Child 3	7.0	4	1.25	4.70	5.95	870.101	404.439	2.151	1.738	22.8
		5	1.55	4.81	6.36	903.339	277.637	3.254	1.538	21.0
mean			1.40	4.75	6.15	886.720	341.038	2.703	1.638	21.9
standard deviation			0.21	0.08	0.29	23.503	89.662	0.779	0.142	1.2
Child 4	11.0	6	0.56	3.34	3.90	1038.780	327.892	3.168	1.100	MD
		7	1.02	10.00	11.02	2280.797	373.143	6.112	0.502	24.4
mean			0.79	6.67	7.46	1659.789	350.517	4.640	0.801	24.4
standard deviation			0.32	4.71	5.04	878.239	31.997	2.082	0.423	
Child 5	12.0	8	0.13	1.98	2.11	2041.027	420.192	4.857	0.147	24.4
		9	0.42	6.75	7.17	2089.348	292.843	7.135	0.110	23.7
mean			0.28	4.36	4.64	2065.188	356.518	5.996	0.129	24.1
standard deviation			0.20	3.38	3.58	34.168	90.050	1.610	0.027	0.5
Child 6	15.0	10	0.33	4.78	5.11	1557.199	397.145	3.921	0.526	MD
		11	0.96	6.18	7.14	2533.497	488.774	5.183	0.178	23.9
mean			0.65	5.48	6.12	2045.348	442.959	4.552	0.352	23.9
standard deviation			0.44	0.99	1.43	690.346	64.792	0.893	0.246	
Child 7	16.0	12	0.42	2.88	3.30	1382.744	306.032	4.518	0.264	23.9
		13	0.67	7.60	8.27	1639.077	322.639	5.080	0.577	22.1
		14	0.53	5.64	6.17	1574.876	407.505	3.865	0.446	22.1
mean			0.54	5.37	5.92	1532.232	345.392	4.488	0.429	22.7
standard deviation			0.13	2.37	2.49	133.381	54.428	0.608	0.157	1.1
mean of mean children	10.1		0.63	4.93	5.57	1725.161	376.228	4.600	0.628	23.7
standard deviation	4.6		0.40	1.30	1.50	472.070	45.508	1.026	0.532	1.3
Elderly adult 1	66.0	15	0.50	2.65	3.16	466.915	274.388	1.702	3.642	25.0
		16	0.23	1.68	1.92	468.605	361.943	1.295	1.904	22.0
		17	0.23	0.88	1.12	562.029	405.288	1.387	2.640	23.1
mean			0.32	1.74	2.06	499.183	347.206	1.461	2.729	23.3
standard deviation			0.16	0.89	1.03	54.433	66.683	0.213	0.872	1.6
Elderly adult 2	75.0	18	0.54	0.85	1.39	578.254	563.182	1.027	6.795	21.7
		19	0.17	1.49	1.66	638.717	643.757	0.992	1.936	21.4
		20	0.28	0.82	1.10	812.017	649.386	1.250	4.671	21.4
		21	0.21	0.67	0.88	580.476	433.933	1.338	3.923	21.4
mean			0.30	0.96	1.26	652.366	572.565	1.152	4.331	21.5
standard deviation			0.17	0.37	0.34	110.054	100.460	0.169	2.008	0.1
Elderly adult 3	96.0	22	0.04	0.43	0.48	512.534	526.430	0.974	6.031	MD
mean of mean elderly adults	79.0		0.22	1.04	1.27	554.694	482.067	1.195	4.364	22.4
standard deviation	15.4		0.16	0.66	0.79	84.849	119.049	0.247	1.651	1.3

Figure(s)
[Click here to download high resolution image](#)



Figure(s)

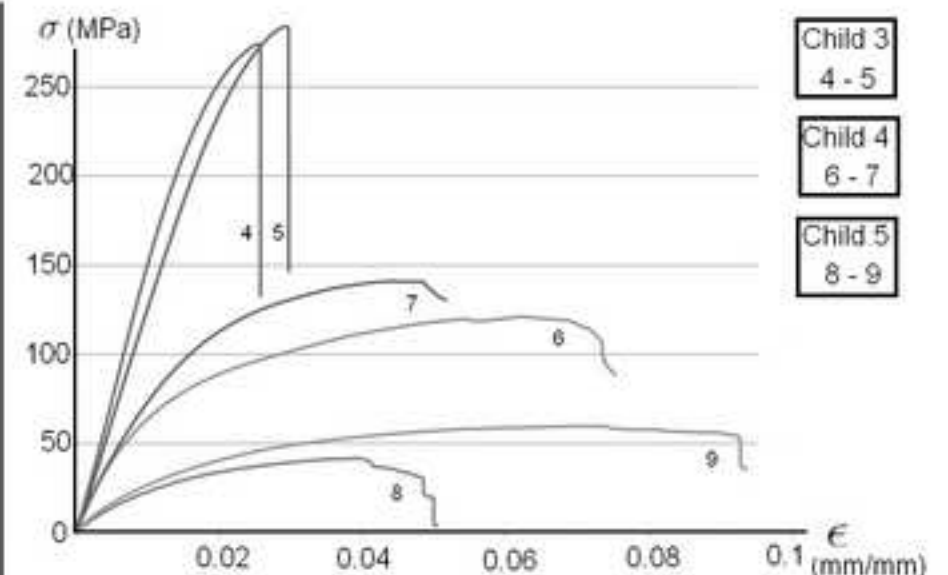
[Click here to download high resolution image](#)



children 5 years old

Child 1
1

Child 2
2-3

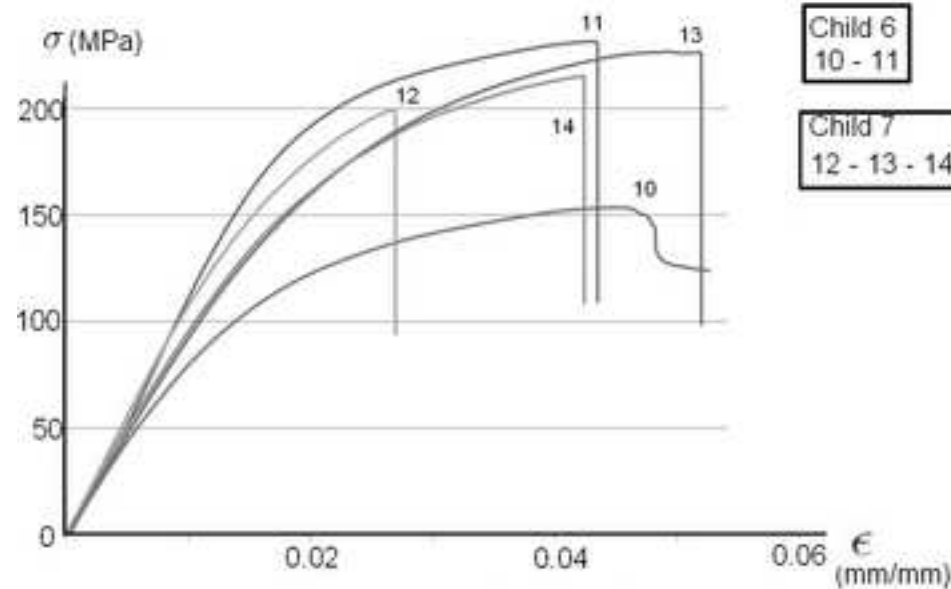


children 7, 11 and 12 years old

Child 3
4-5

Child 4
6-7

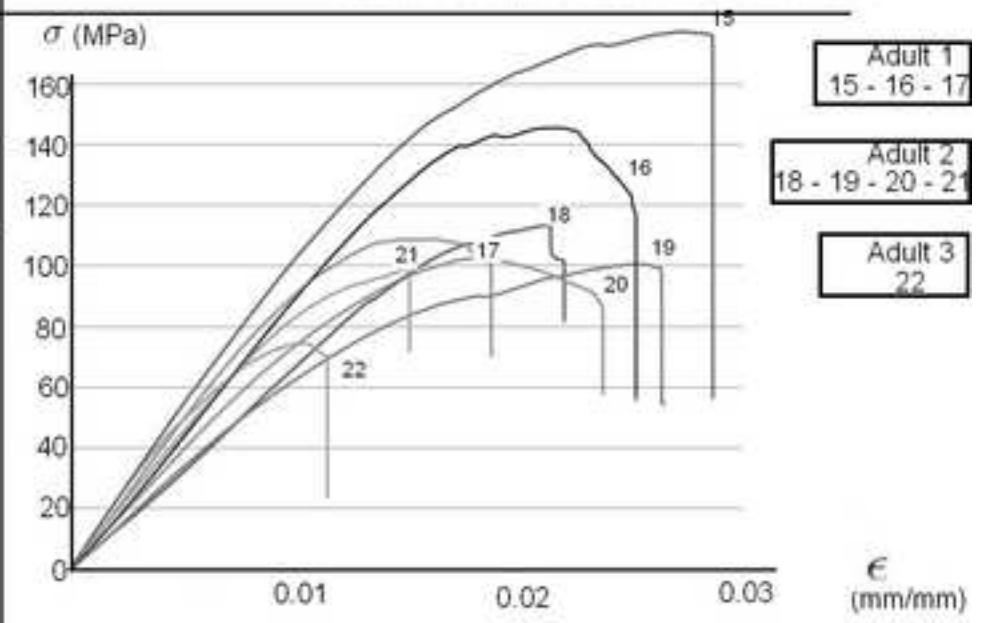
Child 5
8-9



children 15 and 16 years old

Child 6
10-11

Child 7
12-13-14

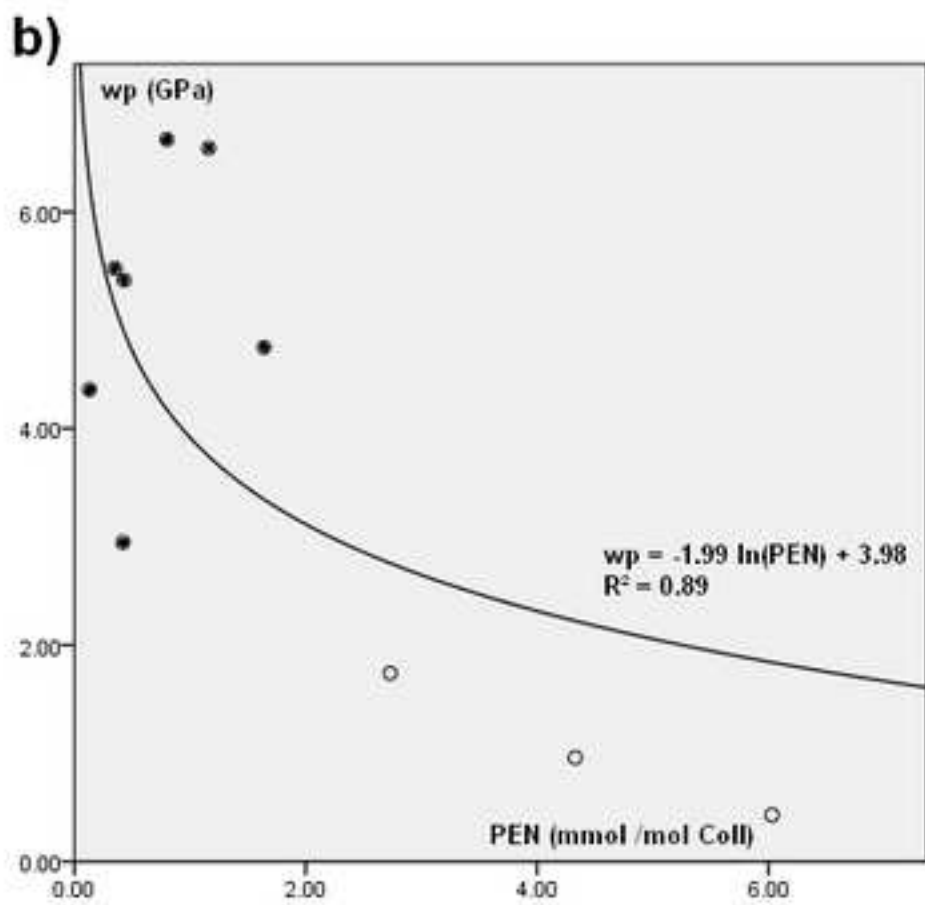
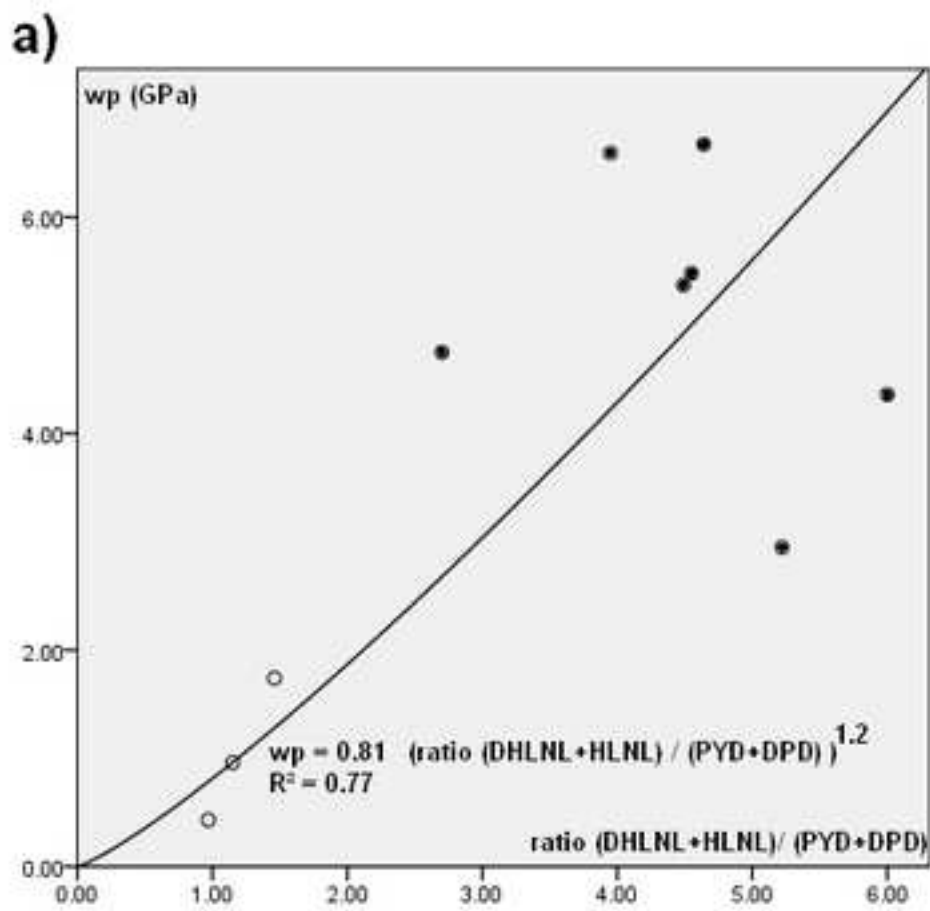


adults

Adult 1
15-16-17

Adult 2
18-19-20-21

Adult 3
22



Figure(s)
[Click here to download high resolution image](#)

