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

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

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ABSTRACT

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

KEY WORDS:

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

Highlights

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

1. INTRODUCTION

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

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

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

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

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

2. MATERIALS AND METHODS

2.1. Bone samples

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

2.2. Mechanical measurements

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

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

2.3. Biochemical measurements

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

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

2.4. Statistical analysis

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

3. RESULTS

3.1. Mechanical measurements

All the stress/strain curves from the microbending test are shown in Figure 2 and toughness data (elastic, plastic and total energies) are listed per sample and per subject (mean) 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 in elderly adults. The ω_p was lower and more clustered in adults compared to children (values more dispersed and higher); a non-parametric negative significant correlation was found 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: ω_p = 0.81 (ratio (DHLNL+HLNL) / (PYD+DPD) ^1.2, R^2 = 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: ω_p = -1.99 ln (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

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

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

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

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

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

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

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

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

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

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

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

5. CONCLUSION

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

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Figure Legends

Figure 1: Microbending test curve (strain/stress) and calculation of energies

Figure 2: Microbending test curves (strain/stress) for all children's and elderly adults' cortical bone samples. Graphs are separate into age, for each range of age. For example, upper left graph represents 3 tests: child number1 has one sample, and children number two has two samples (indicated in the legend, by number 2 & 3).

Figure 3: ω_{tot} as a function of age for elderly adults, for children.

Figure 4: Dependence between ω_p and ratio of (DHLNL + HLNL)/ (PYD + DPD) (a) and PEN (b) for elderly adults' group values (white dots) and children's group values (black dots)

Table legends

Table 1: Age, sex, dimensions, toughness and biochemical measurements of all samples. Mean of toughness and biochemical measurements for each elderly adult and each child (MD 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)

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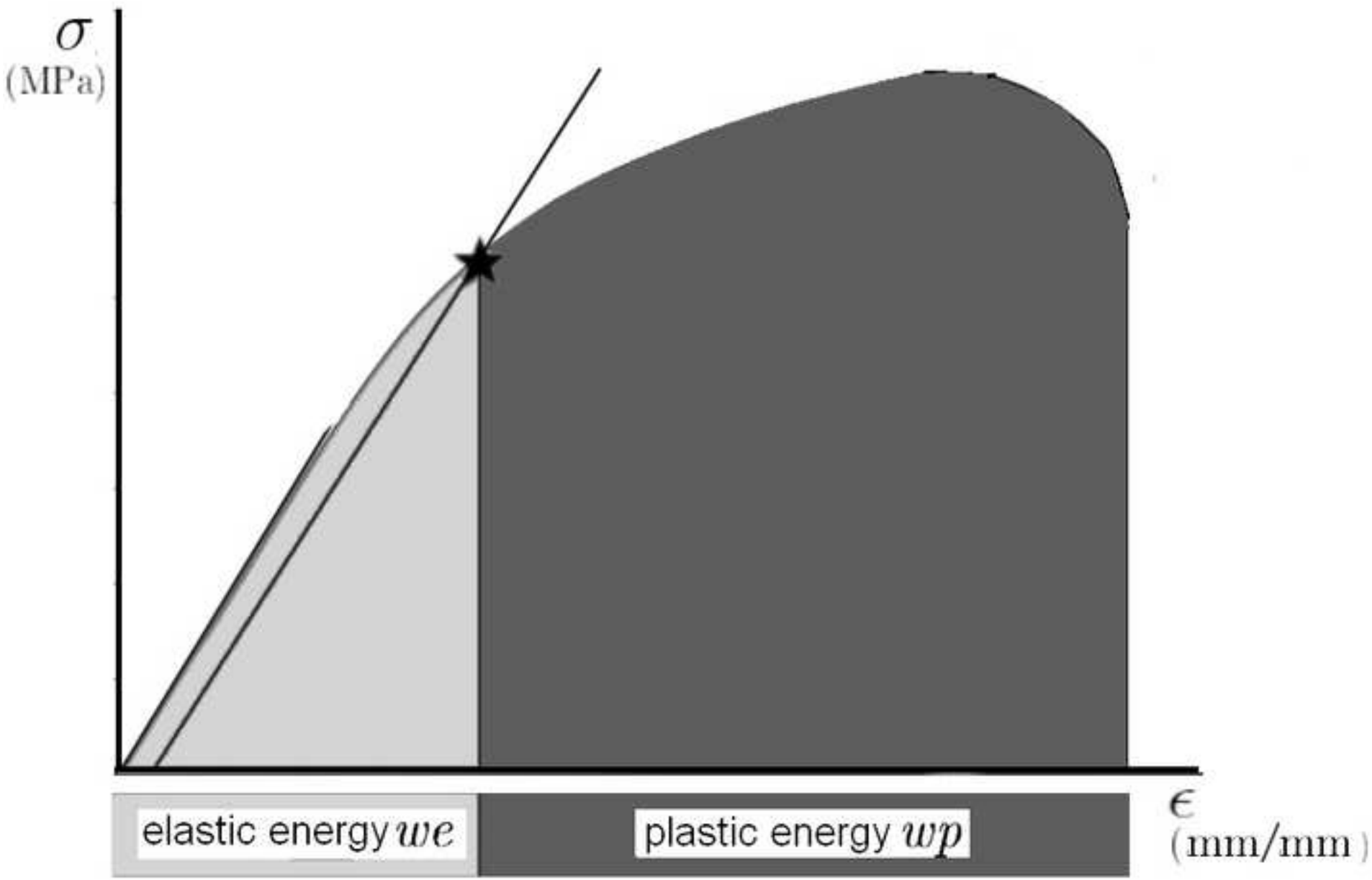

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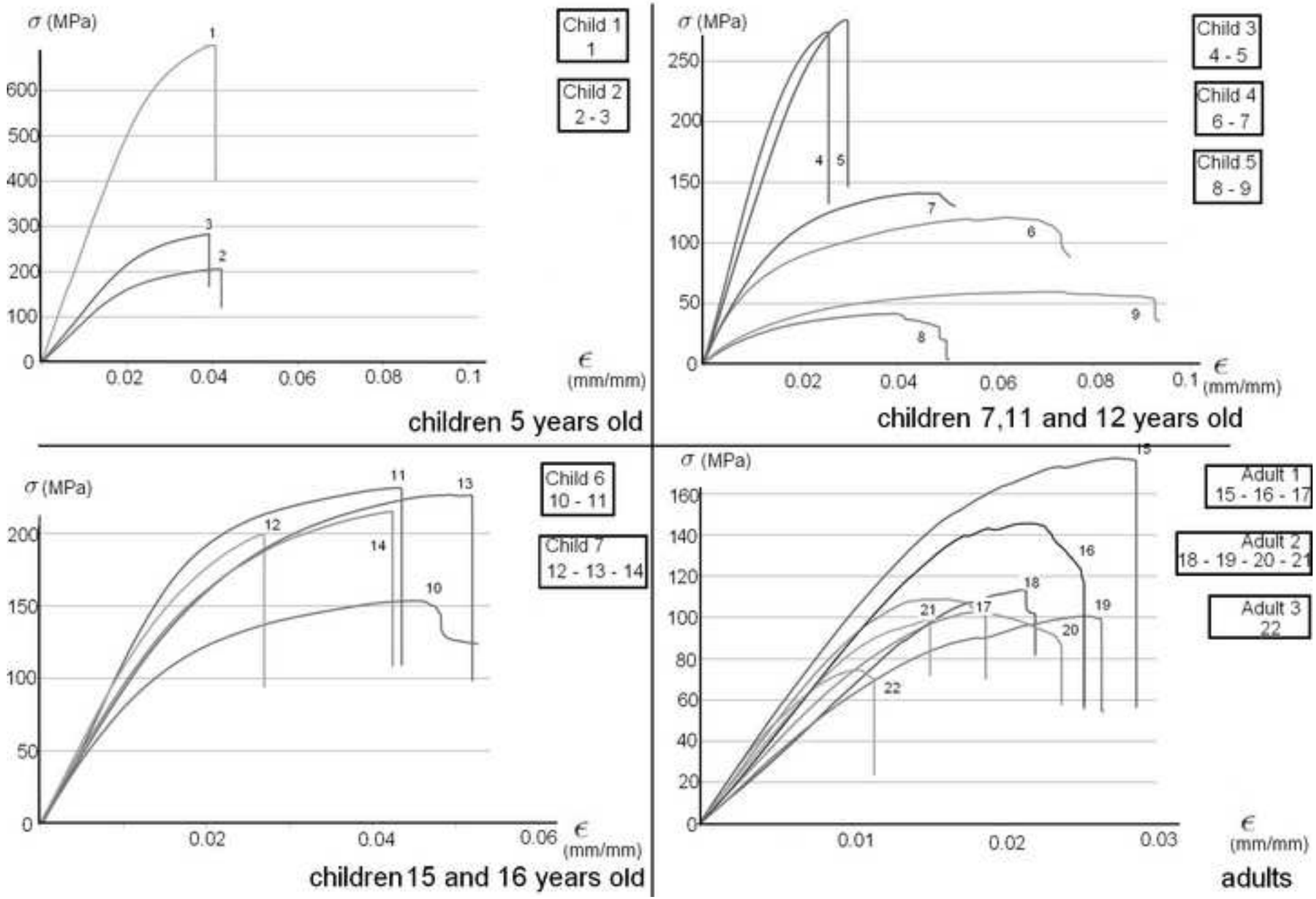

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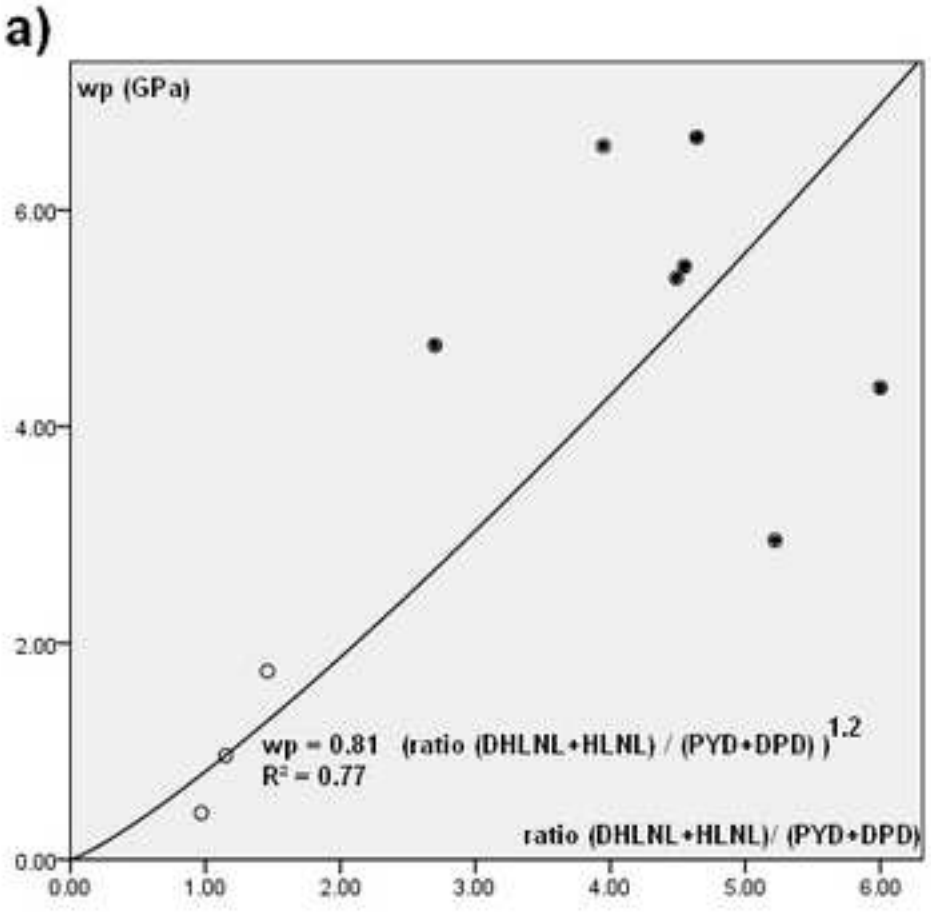

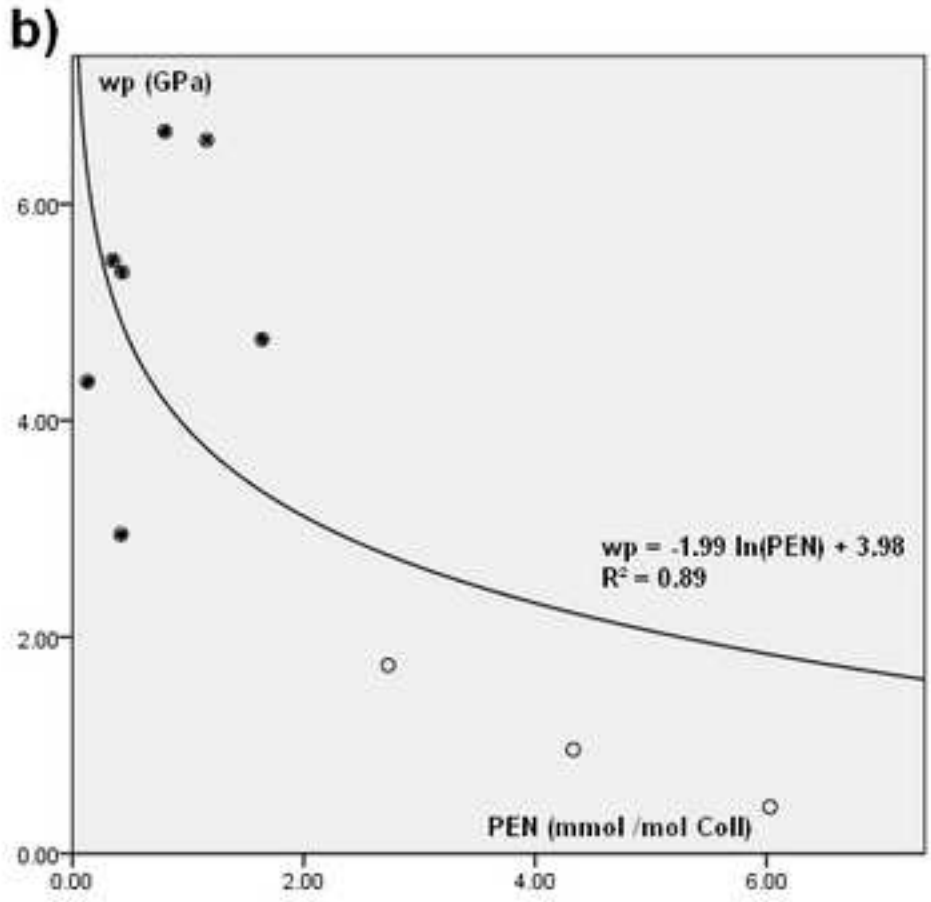

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