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Dermis Mechanical Behaviour after Different Cell Removal Treatments

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Running Head: Dermis Mechanical Behavior for Different Cell Removal Treatments

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30 **Abstract**

31 Human acellular dermal matrices (HADMs) are used in reconstructive surgery as scaffolds
32 promoting autologous tissue regeneration. Critical to the HADM ability to remodel and integrate
33 into the host tissue is the removal of cells while maintaining an intact extracellular architecture.

34 The objective of this work is to develop a methodology to analyse the mechanical properties of
35 HADMs after decellularization to identify its ideal form of treatment and its duration.

36 Two different decellularization techniques were used as a benchmark: the first is a well-
37 established technique (incubation in NaOH for 1 to 7 weeks), and the second is an innovative
38 technique developed by this research group (incubation in DMEM (Dulbecco's modified Eagle
39 medium) for 1 to 7 weeks). After decellularization, the specimens underwent uniaxial tensile tests,
40 and experimental data were represented with stress strain curves, calculating both engineering and
41 true values.

42 Mechanical tests have led to the identification of the optimal method (NaOH or DMEM) and
43 duration for the decellularization treatment; differences between engineering and true values can
44 reach 84%, but the engineering values remain useful to make comparisons, providing reliable
45 indications with a simpler experimental set up and data processing.

46

47 **Keywords** – decellularization treatment, human dermis, static mechanical tests, ultimate stress,
48 ultimate strain, Young's modulus

49

50 **1 Introduction**

51 Engineered skin substitutes have a significant medical practice for patients with extensive burn
52 wounds [1]. Advances in tissue engineering suggest that skin substitutes will be indistinguishable
53 from the normal skin in the near future [2]. However, current skin substitutes do not restore the
54 full native skin physiology because they lack some components such as hair follicles, sebaceous
55 glands and sweat glands [2]. Additionally, the engineered tissue cannot faithfully replicate the
56 mechanical properties of the native skin [1].

57 Currently, alloplastic material and skin allografts, taken from multi-organ donors, are the most
58 suitable integumentary replacement for reconstructive surgery [3]. The immune response to
59 allograft skin is directed primarily against epidermal, endothelial and fibroblast cells in the dermis,
60 while the non-cellular component of the dermis (extracellular matrix) has been demonstrated to
61 be relatively non-immunogenic [4]. Glycerolised acellular alloplastic human dermis (HADM) is
62 used as a matrix for various reconstructive plastic purposes, where it retains almost all of the
63 healthy dermal properties: it is compact and elastic, can be taken into the bed wound, and it retains
64 the intact tissue morphology [5].

65 Different treatments can be used for tissue decellularization [6]. Commonly, a low concentration
66 of NaOH has been used for this aim. The result of this technique is a reliably decellularized matrix.
67 However, surgeons report that this matrix is inferior with reference to handling, ease of use,
68 elasticity and needle penetration resistance. Additionally, decellularization using sodium
69 hydroxide implies the direct contact of the tissue with an aggressive chemical agent, which must
70 necessarily be neutralized by means of incubation in 0.1 N HCl at the end of the decellularization
71 phase. These are the reasons why, in recent times, our research unit has developed an alternative
72 procedure that aims to overcome these limitations. The new methodology consists of keeping the

Dermis Mechanical Behaviour after Different Cell Removal Treatments

73 tissue in DMEM (Dulbecco's modified Eagle medium) for a long period of time (several weeks)
74 while being subjected to mechanical action (tilting). From a biological point of view, the
75 efficiency of the different treatments can be verified by means of an immunohistochemistry
76 analysis, but the preservation of the main mechanical properties of the native dermis also needs
77 to be checked [7]. The aim of this work is to evaluate the mechanical properties of tissue subjected
78 to decellularization treatments varying by type and length to establish the best compromise
79 between a reliably complete decellularization and adequate mechanical properties. The
80 mechanical properties here analysed are the elastic modulus and the ultimate load and strain [8],
81 considering that repaired full-thickness burn wounds may be subject to loss due to dermal
82 substitute deficiencies in tensile strength and elasticity [1] and the requirements of soft-tissue
83 augmentation procedures like rotator cuff [9].

84 The skin is made of three layers, the epidermis, dermis, and hypodermis. It consists of collagen
85 (approximately 75% of the dry weight) and elastin (4% of the dry weight) fibres embedded in a
86 gel-like ground substance consisting of water, small solutes, and macromolecules, predominantly
87 proteoglycans [10]. The dermis provides a major contribution to the overall mechanical
88 characteristics of the skin due to its main constituents, collagen and elastin fibrils, which allow
89 high levels of deformation and flexibility as the fibrils stretch and re-orientate [11]. Collagen
90 fibres are crimped and almost inactive at low strains, while they play a major role at high
91 deformations (where they are stiffer than elastin by approximately three order of magnitude [8]).
92 The skin is anisotropic due to the variable orientation of collagen fibres, with a prevalence along
93 the orientation of the so-called Langer's lines [8]. The dermis can therefore be described as an
94 anisotropic, viscoelastic, nonlinear [12] and non-homogenous material.

95 The tensile test is the most widely used mechanical test performed on *ex vivo* skin specimens.
96 Using this method, the anisotropic, non-linear and viscoelastic behaviours of skin have been
97 explored, as well as its failure properties [13], creep [14], fatigue [15] and preconditioning
98 behaviour [16]. This test is here being used to assess changes in the biomechanical behaviour
99 produced by alterations of the skin's structure, similarly to the approach followed by those authors
100 who studied variations in the collagen content [14] or elastin and proteoglycans contents [10].
101 Due to section narrowing taking place during the specimen loading, different formulations of
102 stress in mechanical tests can produce different results: these are the so called 'nominal' or
103 'engineering values'; their respective 'true' values can be obtained from engineering values under
104 specific assumptions such as volume constancy [17,18]. As true values provide the most faithful
105 representation of the material properties, their estimation requires a complex and demanding
106 experimental set up. This work is also an attempt to quantify differences among these expressions
107 and their limits, establishing if they can or cannot be used for tissue characterization and/or to
108 make comparisons among decellularization treatments.

109 **2 Materials and methods**

110 **2.1 Specimens**

111 Strips of skin tissue, collected from the backs of human donors, were dissected along the cranio-
112 caudal direction. They were decellularized using two different methods based on incubation in
113 0.06 N NaOH or DMEM for 1 to 7 weeks. Immunohistochemistry has been performed for all
114 treatments to verify the decellularization, according to the following procedure. Biopsy samples
115 were washed in physiological solution, fixed in 4% neutral-buffered formalin and embedded in
116 formalin by routine processing (FFPE). FFPE samples were sectioned at a thickness of 2-3 μm

117 for immunohistochemistry reactions, and immunohistochemistry was performed using an
118 automated slide-processing platform (Ventana BenchMarkXT Autostainer, Ventana Medical
119 Systems, Tucson, AZ, USA).” HADMs, preserved at 85% glycerol in a 4°C refrigerator at the
120 Turin Skin Bank (Italy) and unfit for transplantation, were used for these experiments after the
121 approval of the Institutional Ethical Board of *Azienda Ospedaliera Universitaria Città della*
122 *Salute e della Scienza* of Turin, Italy, (approved on January 23rd, 2012 with protocol number
123 0006730), and written informed consent was obtained from all study participants. Before use, the
124 dermis grafts were washed to remove all of the glycerol, dipping them sequentially in three
125 different beakers filled with abundant saline solution 0.9% at +37°C for more than three minutes
126 each, as prescribed by the Euro Skin Bank [19]. The specimens were obtained by cutting out
127 approximately 2x4 mm strips along the cranio-caudal (CC) and medio-lateral (ML) directions
128 using a custom made die cutter; this cutting method avoids generating notches and defects that
129 could bias tests. The resulting specimen sizes were measured by means of photogrammetry before
130 mechanical testing: 4.33 ± 0.57 -mm width, 2.21 ± 0.32 -mm thickness, 10.10 ± 0.38 -mm length
131 (average \pm std).

132 On the whole, there were 3–4 specimens (depending on the original strip size and shape) for each
133 combination of decellularization method (NaOH or DMEM), duration (called ‘Tx’ in the
134 following, where x represents the number of weeks of incubation) and cut orientation (CC or ML),
135 for a total 96 specimens. Intact human skin was used as a control (called ‘T0’ in the following, as
136 it did not undergo any decellularization treatment).

137 **2.2 Photogrammetry set-up**

138 Two different photographic set-ups have been developed to measure the specimens. The first was
139 finalized to measure the specimens’ size at rest and was made of a full-frame digital camera

140 (Canon EOS 5D Mark II) with an autofocus lens for macro photography (Canon EF 100 mm f/2.8
141 Macro USM), a camera stand with two light stands, and a tripod. A second set-up was developed
142 to follow tensile tests; it included the previously described digital camera as well as a second
143 digital single-lens reflex camera (Canon EOS 400D). When the two cameras were triggered, they
144 acquired the frontal and lateral views of the specimen through a remote capture software (DSLR
145 Remote Pro). The width and the thickness of the specimens were measured using the image
146 analysis software ImageJ (National Institutes of Health, Bethesda, Maryland, U.S.) as an average
147 of five different measurements, reaching a 0.01 mm/pixel measurement resolution given a 21.0
148 MP image (5616x3744 pixels).

149 **2.3 Mechanical tests**

150 Samples were subjected to uniaxial tensile tests along both the cranio-caudal and medio-lateral
151 directions to quantify the influence of the chemical treatment on the skin tissue's biomechanical
152 behaviour. Testing parameters have been set according to the physiological loads, the expected
153 tissue behaviour, and the Bose Electroforce® features. For example, the strain rate could reach
154 very high values in reality due to impact forces, but the characteristics of the material are strain
155 rate dependent [20], and the test speed had to be limited to 3.2%/s so as not to exceed the load
156 cell range and risking rupture. The specimen length also had to be chosen considering the
157 physiologic peak strain (over 100%) and the machine stroke (± 6 mm), together with the limited
158 sample extension; these considerations led to the selection of a ~~10~~5 mm specimen length. The
159 specimens were clamped by titanium machine grips that were specifically developed for
160 biomaterials and have knurled-flat faces to prevent slipping. The analysis of the video recordings
161 demonstrates that there were neither anomalous behaviours nor failures near the clamps. Sliding
162 through the testing grips was excluded, too, as no abrupt increase or decrease was detected in the

163 experimental curves. No marks were observed on the specimen ends, and the extension of the
164 grasped ends was found to be unchanged.

165 Up to the instant preceding the tensile test, all specimens were kept hydrated in physiological
166 solution; no additional hydration was carried out during the test due to the absence of a
167 thermostatic bath. This was not judged to be a major shortcoming because the tests lasted less
168 than one minute. Specimens were constrained to the Bose Electroforce® testing machine,
169 clamping their ends along the longitudinal direction.

170 No preconditioning cycles were performed because the dermal tissue is a bi-phasic structure, like
171 most soft tissues, and preconditioning has been demonstrated to significantly influence the
172 mechanical response of these tissues. Slow viscoelastic phenomena related to fluid flow initiate
173 starting from the very first loading cycles, so the final mechanical properties would depend on the
174 pre-conditioning protocol [21].

175 The testing room temperature was 20° C, while the humidity ranged between 40 and 65%. The
176 displacement was set equal to zero when a 0.05 N force was recorded.

177 Rupture tensile tests were performed for all samples in displacement control at a strain rate of
178 0.032 s⁻¹. The initial gap between the grips was 5 mm.

179 **2.4 Data Elaboration**

180 The results of rupture tests on soft tissues are often reported in terms of ‘engineering’ stress and
181 strain in the literature, with a few exceptions where the specimen section is monitored during
182 tests, and the strain distribution is assessed by full-field techniques [17,22]. In this work, the
183 engineering and true values have been calculated, as detailed in the following.

184 The "engineering curve" is obtained by ignoring the narrowing of the section during the elongation
185 of the sample and referring always to the initial specimen length. The engineering stress σ_e (Eq.

186 1) is therefore calculated by dividing the force F by the unloaded-cross sectional area A_0 of the
 187 specimen; the engineering strain ϵ_e (Eq. 2) is expressed as the change in length ΔL per unit of the
 188 original length L_0 . It should be emphasised that the measurement of the engineering strain would
 189 require a dog-bone shaped specimen and a calibrated length whose elongation is monitored, while
 190 a rectangular specimen has been here used and its elongation has been evaluated on the basis of
 191 the clamp-to-clamp displacement; the authors considered that this was not a hard limitation due
 192 to the high compliance of the tissue, which “homogenises” the stress field (see, for example, the
 193 work of Taylor et al. on crack propagation [23]). The engineering Young’s modulus (E_e) has been
 194 calculated from the linear portion of the stress-strain curve [8], which is the so-called ‘linear
 195 region’ where collagen chains are stretched [12,24]: curve data were locally derived with a
 196 moving average linear regression, and the constant trend of the derived curve was considered.

197 The true stress σ_t is the ratio between the force and the minimum section A_{min} ; it is approximately
 198 coincident with the engineering curve, up to the strain where section narrowing becomes
 199 conspicuous. The true curve can be obtained by monitoring the neck area during the tensile test:
 200 the history of the section variation $A_{min}(t)$ needs to be acquired, monitoring both the specimen
 201 width $b_{min}(t)$ and thickness $s_{min}(t)$ at the neck region. In the literature, an alternative expression for
 202 the true stress is often used, which relies on the hypothesis of a null variation of the specimen
 203 volume [25]: this expression is simpler to be implemented because it requires only the estimation
 204 of the real-time specimen length (like for the engineering curve). The respective value σ_{st} will be
 205 called the ‘simplified true’ stress, and it can be obtained from the engineering curve by analytical
 206 transformations ($\sigma_{st} = \sigma_e \cdot (1 + \epsilon_e)$). The corresponding ‘simplified true’ elastic modulus E_{st} can be
 207 calculated on σ_{st}/ϵ_e curves.

208 The evaluation of the true Young's modulus E_t has been performed on the basis of the acquired
 209 force and displacement signals and of the specimen shape; given a certain force F , the specimen
 210 volume can be divided axially into infinitesimal portions dy whose section is $A(y,F)$. Therefore,
 211 the whole specimen elongation ΔS_{ab} in the linear portion of the force/displacement curve (a, b,
 212 figure 1) can be expressed as

$$213 \quad \Delta S_{ab} = \int_{F_a}^{F_b} \int_0^l ds = \int_{F_a}^{F_b} \int_0^l \varepsilon_t \cdot dy = \int_{F_a}^{F_b} \left[\int_0^l \frac{1}{E_t \cdot A(y,F)} dy \right] dF = \frac{1}{E_t} \int_{F_a}^{F_b} \left[\int_0^l \frac{dy}{A(y,F)} \right] dF$$

214 where the Young's modulus has been considered to be linear (independent of the force level) and
 215 constant all over the specimen, as it should be in the above-mentioned 'linear elastic region'. This
 216 formula could not be used up to the failure region (to obtain the true ultimate strain, for example).
 217 The 'true' Young's modulus can be so derived:

$$218 \quad E_t = \frac{\int_{F_a}^{F_b} \left[\int_0^l \frac{dy}{A(y,F)} \right] dF}{\Delta S_{ab}}$$

219 The numerator requires the knowledge of the section variation for each force step, and at different
 220 quotes (y), and it can be estimated thanks to the photogrammetry set up.

221 A number of descriptive parameters can be so obtained: the ultimate tensile strength (UTS, UTS_i,
 222 UTS_{st}), the ultimate deformation ($\varepsilon_{UTS,e}$), and the Young's modulus (E_e , E_{st} , E_t). True values have
 223 been calculated only for those decellularization treatments that produced 'engineering' and
 224 'simplified true' mechanical properties similar to those of the native dermis ($p < 0.05$, Tukey-
 225 Kramer test, as detailed in the following).

226 **2.5 Statistical analysis**

227 The mechanical properties of the dermis were reported in relation to the testing direction (CC or
 228 ML), the type of decellularization treatment (called NaOH or DMEM in the following), and the

229 duration of the treatments (from 0 to 7 weeks at 1 week steps, called T0, T1 T7 in the
230 following).

231 The statistical analysis of the experimental results was carried out using a multivariate analysis of
232 variance (Matlab function 'anovan'), followed by a Tukey–Kramer post hoc test, after having
233 tested the normality of the statistical distribution of all parameters by the Lilliefors test function.
234 Significance levels were set to $p < 0.05$ for all tests.

235 **3 Results**

236 The analysis of video recordings demonstrated that there were neither anomalous behaviours nor
237 failures near clamps; therefore, all acquired data have been elaborated.

238 Figures 2 shows typical stress/strain curves for the engineering, simplified true and true
239 formulations. Dealing with the ultimate stress (Fig. 3-5), the engineering stress leads to
240 underestimate the UTS by up to -71% and the Young's modulus by up to -84%. The simplified
241 true stress would underestimate the UTS by up to -44%. The error coming from the simplified
242 true stress evaluation demonstrates how the hypothesis that the section variation is inversely
243 proportional to the longitudinal strain (equivalent to the 'constant volume' hypothesis for small
244 deformations) does not hold: this is not surprising because in the literature, both analytical and
245 experimental demonstrations of the soft tissue volume variation during tensile tests can be found
246 [26,27]).

247 All sample properties are shown to be normally distributed, according to the Lilliefors test
248 ($p < 0.05$), so the following variance analysis could be performed.

249 The results of the analysis of variance are shown in Table 1: the type of treatment, its duration,
250 and the specimen orientation are all significant factors, as is their interaction ($p < 0.05$), with the

251 only exception of the specimen orientation for the ultimate strain. The mechanical behaviour
252 along the CC direction is significantly stiffer compared to that in the ML direction, and the
253 mechanical strength is higher (+77.1% E_e , +46.6% UTS_e , -16.1% $\epsilon_{UTS,e}$, figures 3-5). DMEM
254 treatment is generally less aggressive than NaOH treatment (figures 3-5), and the mechanical
255 properties do not vary monotonously over the treatment length (figures 3-5).

256 A more detailed statistical analysis has been undertaken to establish which factor levels produced
257 significantly different results compared to reference groups (respectively, T0-CC and T0-ML) by
258 means of Tukey-Kramer tests, aiming to identify the best treatment type and duration as the
259 combination producing the results most similar to those of native tissue. Looking at Figures 3-5,
260 only minor differences exist between the engineering and 'simplified true' formulation results,
261 and some general conclusions could be drawn. The tissue properties along the CC direction
262 significantly degrade (lower UTS and E) for all treatments and durations, with $\epsilon_{UTS,e}$ being the
263 only mechanical property that is not affected significantly. In the ML direction, T0, DMEM T5,
264 DMEM T6, DMEM T7, and NaOH T5 produce similar mechanical properties, according to both
265 the engineering and simplified true formulations. These same treatments have been further
266 investigated to assess if the true stress formulation would lead to the same conclusions. DMEM
267 T5, DMEM T5, NaOH T5, and, partly NaOH T6 (assuming $p=0.03$) still produced mechanical
268 properties close to those of native tissue for samples cut along the ML direction.

269 Native specimens cut along the CC direction continued to show a higher Young's modulus E_t and
270 UTS_t ; no treatment for any duration could preserve these properties.

271 **4 Discussion**

272 The native skin from which the HADM scaffold is prepared must be mechanically or physically
273 separated from unwanted tissue and cell structures, and this processing step could alter the
274 integrity and the architecture of the matrix and, in turn, influence the mechanical and material
275 properties of the matrix. The efficiency of cell removal from a tissue is dependent on the origin
276 of the tissue and the specific physical, chemical, and enzymatic methods that are used [28]. A
277 similar consideration holds for the mechanical properties of the scaffold, as demonstrated in this
278 work.

279 Experimental tests were performed at 20 °C, so the measured properties cannot be immediately
280 converted to physiological properties at 37°C. The reason for this choice is the simplification of
281 the experimental set up and being able to compare these results with most works in the literature
282 in which mechanical tests have been carried out at ‘room temperature’ [8,11,15,29,17,18,30]. The
283 results of the experimental tests were compared, assuming a perfectly uniaxial loading condition
284 and a uniform distribution of collagen fibres. This is a limit in the present experimental set up, as
285 the specimen is rectangular and its contraction is not allowed at the machine clamps, so the
286 uniaxial stress hypothesis is not verified at the specimen ends. Using dog-bone shaped specimens
287 would not completely solve this issue: in the case of longitudinal samples with most collagen
288 fibres oriented axially, it would make no difference because the interrupted fibres (those placed
289 more laterally) would be inactive. Longer specimens would have minimised the influence of the
290 clamped ends, but they would have limited the maximum strain because the employed loading
291 machine allows 12 mm displacement at the most. Finally, it should be stressed that the notch
292 sensitivity in soft tissues is very low [23], so a minor area on the specimen is likely to be affected
293 by the clamps. Ongoing numerical tests are confirming these hypotheses (nonlinear analysis, with
294 large displacements, fig. 6), but the full strain field should be experimentally acquired as a final

295 validation. This is a quite demanding experimental set-up. Some authors are setting up systems
296 based on digital image correlation [22]; this is certainly a promising technique that deserves to be
297 considered in future tests on biological tissues.

298 Results have been here expressed through engineering, simplified true and true curves because
299 the results of rupture tests for soft tissues have not always been reported in a standard manner in
300 the literature [18]. Dealing with comparisons among different treatment types and durations and
301 sample directions, all three representations produced substantially similar results.

302 A review of decellularization methods [6] agrees with the results here obtained regarding the
303 NaOH cell removal treatment. In fact, it stated that bases are harsh, so are commonly used to
304 eliminate growth factors from the matrix, even though they decrease ECM mechanical properties
305 more significantly than chemical and enzymatic agents. In this work, the NaOH treatment has
306 been proven to weaken the mechanical properties of the tissue, especially with reference to the
307 cranio-caudal direction. The primary mechanism by which bases such as sodium hydroxide reduce
308 the mechanical properties is the cleavage of collagen fibrils and disruption of collagen crosslinks.
309 Richters *et al.* [31] evaluated a cost-effective method based on low concentrations of NaOH for
310 the decellularization of human donor skin preserved in 85% glycerol, and they found that a 6 week
311 incubation period was optimal, as stated in the present work, while longer periods caused damage
312 to the collagen fibres, although the elastin fibres appeared to be well preserved, and this could
313 explain the different behaviours observed along the cranio-caudal and medio-lateral directions.

314 DMEM coupled to mechanical action has been used as a cell removal treatment for the first time
315 in this work, so similar tests cannot be found in the literature. Other decellularization methods
316 include a wide variety of chemicals, but if the chemicals remain within the tissue in high
317 concentrations after treatment, they can potentially invoke an adverse immune response by the

318 host (see, for example, enzymes commonly derived from bovine sources such as DNase, RNase,
319 and trypsin). Herein, one of the most simple decellularization methods was studied (long-term
320 incubation in culture medium), and preliminary immunohistochemical and histological results
321 (unpublished data) demonstrate the complete decellularization of the tissue. DMEM treatment has
322 also proven to be more conservative with reference to the medio-lateral direction because the
323 mechanical properties of specimens treated with DMEM are generally higher than those measured
324 on specimens treated with NaOH for the same number of weeks.

325 From a biological point of view, both DMEM and NaOH show, in the immunohistochemical
326 evaluation, a good decellularization of grafts after only 4 weeks of treatment. However, the
327 DMEM-treated samples exhibit better handling, greater flexibility and lower needle penetration
328 resistance, according to surgeons' evaluations, and are therefore preferable. Additionally, the
329 DMEM treatment avoids the use of chemical agents, as opposed to NaOH, which needs to be
330 neutralized at the end of the decellularization process. Therefore, DMEM is less likely to produce
331 inflammatory responses.

332 The objective of this work was to set up a procedure to perform biomechanical comparisons
333 among decellularization treatments; the complete quantification of the skin's anisotropic
334 behaviour would require a greater number of samples, from different donors, and biaxial testing.

335 This experimental set up can allow only the measurement of the Young's modulus and failure
336 properties along two reference orthogonal directions (parallel and perpendicular to the Langer's
337 lines [8]). Nevertheless, in the following, a comparison with results obtained from other authors
338 [8,32,33] is reported to verify the differences that exist and how they can be justified (Table 2).

339 Ni Annaidh *et al.* [8] reported force–displacement curves for each tensile test performed and
340 calculated the engineering stress and strain. Their standard deviations were much larger; the

341 average coefficients of variation (ratios of the standard deviation to the mean) are up to 0.80 for
342 UTS and 0.97 for E, against the values obtained in this work, 0.09 and 0.10, respectively, due to
343 the number of specimens and the specimens having been taken from several donors (Table 2).
344 The values calculated in this work are most similar to those obtained on the ‘lower back’ and are
345 generally lower (up to -43% for UTS, up to -46% for ϵ_{UTS} , up to -68% for E) compared to those
346 reported in [8]. This can be explained by the smaller size of the specimens, which results in more
347 severe striction and consequently lower nominal stresses.

348 Yoder and Elliott [32] characterized human allografts by considering the engineering stress and
349 calculated two-dimensional Lagrangian strains from optical images using Vic2D software. The
350 Young's modulus (Table 2) compares favourably to the results here reported for DMEM and
351 NaOH at T5 or T6 for engineering curves with reference to the ML direction. A 20 times higher
352 E along the ‘parallel’ direction is reported in [32]; this result is against the findings of this work
353 and [8], which both report a lower level of anisotropy in tested tissues.

354 Up to now, the failure properties and the elastic behaviour for static loads has been investigated,
355 as critical aspects of dermal patches include stiffness mismatch [35] and the eventual failure.
356 Nevertheless, cyclic loading parameters also need to be considered because in a highly
357 collagenous tissue such as skin, the elastic recoil and hysteresis of the material would be of utmost
358 importance.

359 **References**

360

- 361 [1] Sander EA, Lynch KA, Boyce ST. Development of the mechanical properties of engineered
362 skin substitutes after grafting to full-thickness wounds. *J Biomech Eng* 2014;136:051008.
363 doi:10.1115/1.4026290.
364

Dermis Mechanical Behaviour after Different Cell Removal Treatments

- 365 [2] Sun BK, Saprashvili Z, Khavari PA. Advances in skin grafting and treatment of cutaneous
366 wounds. *Science* 2014;346:941–5. doi:10.1126/science.1253836.
367
- 368 [3] Wong DJ, Chang HY. *Skin tissue engineering*. Harvard Stem Cell Institute; 2009.
369
- 370 [4] Badylak SF, Freytes DO, Gilbert TW. Reprint of: Extracellular matrix as a biological
371 scaffold material: Structure and function. *Acta Biomater* 2015;23:S17–26.
372 doi:10.1016/j.actbio.2015.07.016.
373
- 374 [5] Deeken CR, Eliason BJ, Pichert MD, Grant SA, Frisella MM, Matthews BD.
375 Differentiation of biologic scaffold materials through physicochemical, thermal, and
376 enzymatic degradation techniques. *Ann Surg* 2012;255:595–604.
377 doi:10.1097/SLA.0b013e3182445341.
378
- 379 [6] Crapo PM, Gilbert TW, Badylak SF. An overview of tissue and whole organ
380 decellularization processes. *Biomaterials* 2011;32:3233–43.
381 doi:10.1016/j.biomaterials.2011.01.057.
382
- 383 [7] Butler DL, Goldstein SA, Guilak F. Functional tissue engineering: the role of
384 biomechanics. *J Biomech Eng* 2000;122:570–5.
385
- 386 [8] Ní Annaidh A, Bruyère K, Destrade M, Gilchrist MD, Otténio M. Characterization of the
387 anisotropic mechanical properties of excised human skin. *J Mech Behav Biomed Mater*
388 2012;5:139–48. doi:10.1016/j.jmbbm.2011.08.016.
389
- 390 [9] Moore MA, Samsell B, Wallis G, Triplett S, Chen S, Jones AL, et al. Decellularization of
391 human dermis using non-denaturing anionic detergent and endonuclease: a review. *Cell*
392 *Tissue Bank* 2015;16:249–59. doi:10.1007/s10561-014-9467-4.
393
- 394 [10] Eshel H, Lanir Y. Effects of strain level and proteoglycan depletion on preconditioning and
395 viscoelastic responses of rat dorsal skin. *Ann Biomed Eng* 2001;29:164–72.
396
- 397 [11] Sanders R. Torsional elasticity of human skin in vivo. *Pflugers Arch* 1973;342:255–60.
398
- 399 [12] Fung YC. *Biomechanics - Mechanical Properties of Living Tissues* | Y. C. Fung | Springer.
400 2nd ed. New York: Springer-Verlag; 1993.
401
- 402 [13] Pereira BP, Lucas PW, Swee-Hin T. Ranking the fracture toughness of thin mammalian

- 403 soft tissues using the scissors cutting test. *J Biomech* 1997;30:91–4.
404
- 405 [14] Del Prete Z, Antonucci S, Hoffman AH, Grigg P. Viscoelastic properties of skin in Mov-
406 13 and Tsk mice. *J Biomech* 2004;37:1491–7. doi:10.1016/j.jbiomech.2004.01.015.
407
- 408 [15] Muñoz MJ, Bea J a., Rodríguez JF, Ochoa I, Grasa J, Pérez del Palomar a., et al. An
409 experimental study of the mouse skin behaviour: Damage and inelastic aspects. *J Biomech*
410 2008;41:93–9. doi:10.1016/j.jbiomech.2007.07.013.
411
- 412 [16] Liu Z, Yeung K. On Preconditioning and Stress Relaxation Behaviour of Fresh Swine Skin
413 in Different Fibre Direction. *Biomed Pharm Eng 2006 ICBPE 2006 Int Conf 2006*:221–6.
414
- 415 [17] Jacquemoud C, Bruyere-Garnier K, Coret M. Methodology to determine failure
416 characteristics of planar soft tissues using a dynamic tensile test. *J Biomech* 2007;40:468–
417 75. doi:10.1016/j.jbiomech.2005.12.010.
418
- 419 [18] Khanafer K, Schlicht MS, Berguer R. How Should We Measure and Report Elasticity in
420 Aortic Tissue? *Eur J Vasc Endovasc Surg* 2013;45:332–9. doi:10.1016/j.ejvs.2012.12.015.
421
- 422 [19] Euro Tissue Bank - Manual n.d. [http://www.eurotissuebank.nl/euro-skin-bank-huidbank-](http://www.eurotissuebank.nl/euro-skin-bank-huidbank-esb-en-GB/what-is-donor-skin/manual/)
423 [esb-en-GB/what-is-donor-skin/manual/](http://www.eurotissuebank.nl/euro-skin-bank-huidbank-esb-en-GB/what-is-donor-skin/manual/) (accessed October 14, 2015).
424
- 425 [20] Arumugam V, Naresh MD, Sanjeevi R. Effect of strain rate on the fracture behaviour of
426 skin. *J Biosci* 1994;19:307–13. doi:10.1007/BF02716820.
427
- 428 [21] Hosseini SM, Wilson W, Ito K, van Donkelaar CC. How preconditioning affects the
429 measurement of poro-viscoelastic mechanical properties in biological tissues. *Biomech*
430 *Model Mechanobiol* 2013;503–13. doi:10.1007/s10237-013-0511-2.
431
- 432 [22] Bel-Brunon A, Kehl S, Martin C, Uhlig S, Wall WA. Numerical identification method for
433 the non-linear viscoelastic compressible behavior of soft tissue using uniaxial tensile tests
434 and image registration - application to rat lung parenchyma. *J Mech Behav Biomed Mater*
435 2014;29:360–74. doi:10.1016/j.jmbbm.2013.09.018.
436
- 437 [23] Taylor D, O’Mara N, Ryan E, Takaza M, Simms C. The fracture toughness of soft tissues.
438 *J Mech Behav Biomed Mater* 2012;6:139–47. doi:10.1016/j.jmbbm.2011.09.018.
439
- 440 [24] Özkaya N, Nordin M, Goldsheyder D, Leger D. Fundamentals of Biomechanics -

Dermis Mechanical Behaviour after Different Cell Removal Treatments

- 441 Equilibrium, Motion, and | Nihat Özkaya | Springer. 3rd ed. New York: Springer-Verlag;
442 2012.
443
- 444 [25] Silver FH, Freeman JW, DeVore D. Viscoelastic properties of human skin and processed
445 dermis. *Skin Res Technol* 2001;7:18–23.
446
- 447 [26] Adeeb S, Ali A, Shrive N, Frank C, Smith D. Modelling the Behaviour of Ligaments: A
448 Technical Note. *Comput Methods Biomech Biomed Engin* 2004;7:33–42.
449 doi:10.1080/10255840310001637266.
450
- 451 [27] Reese SP, Maas SA, Weiss JA. Micromechanical models of helical superstructures in
452 ligament and tendon fibers predict large Poisson's ratios. *J Biomech* 2010;43:1394–400.
453 doi:10.1016/j.jbiomech.2010.01.004.
454
- 455 [28] Chen R-N, Ho H-O, Tsai Y-T, Sheu M-T. Process development of an acellular dermal
456 matrix (ADM) for biomedical applications. *Biomaterials* 2004;25:2679–86.
457 doi:10.1016/j.biomaterials.2003.09.070.
458
- 459 [29] Li L, Qian X, Wang H, Hua L, Zhang H, Liu Z. Power type strain energy function model
460 and prediction of the anisotropic mechanical properties of skin using uniaxial extension
461 data. *Med Biol Eng Comput* 2013;51:1147–56. doi:10.1007/s11517-013-1098-6.
462
- 463 [30] Ní Annaidh A, Bruyère K, Destrade M, Gilchrist MD, Maurini C, Otténio M, et al.
464 Automated Estimation of Collagen Fibre Dispersion in the Dermis and its Contribution to
465 the Anisotropic Behaviour of Skin. *Ann Biomed Eng* 2012;40:1666–78.
466 doi:10.1007/s10439-012-0542-3.
467
- 468 [31] Richters CD, Pirayesh A, Hoeksema H, Kamperdijk EWA, Kreis RW, Dutrieux RP, et al.
469 Development of a dermal matrix from glycerol preserved allogeneic skin. *Cell Tissue Bank*
470 2008;9:309–15. doi:10.1007/s10561-008-9073-4.
471
- 472 [32] Yoder JH, Elliott DM. Nonlinear and anisotropic tensile properties of graft materials used
473 in soft tissue applications. *Clin Biomech* 2010;25:378–82.
474 doi:10.1016/j.clinbiomech.2010.01.004.
475
- 476 [33] Edwards C, Marks R. Evaluation of biomechanical properties of human skin. *Clin*
477 *Dermatol* 1995;13:375–80. doi:10.1016/0738-081X(95)00078-T.
478
- 479 [34] Daly CH. Biomechanical properties of dermis. *J Invest Dermatol* 1982;79 Suppl 1:17s –
19

Dermis Mechanical Behaviour after Different Cell Removal Treatments

480 20s.
481

482 [35] Hopp I, Michelmore A, Smith LE, Robinson DE, Bachhuka A, Mierczynska A, et al. The
483 influence of substrate stiffness gradients on primary human dermal fibroblasts.
484 *Biomaterials* 2013;34:5070–7. doi:10.1016/j.biomaterials.2013.03.075.
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489 **Table 1: Anova results for the ultimate stress, ultimate strain and elastic modulus with reference to**
 490 **the engineering formulation. Boldface characters are used to highlight factors that are not significant**
 491 **($p > 0.05$)."**
 492

<i>Ultimate Stress</i>					
Source	Sum Sq.	DOF	Mean Sq.	F	p
Treatment	30.90	1	30.90	128.22	6.28E-17
Orientation	11.00	1	11.00	45.67	4.94E-09
Duration	257.24	7	36.75	152.50	2.08E-37
Treatment*Orientation	12.04	1	12.04	49.97	1.41E-09
Treatment*Duration	13.57	7	1.94	8.05	5.45E-07
Orientation*Duration	64.42	7	9.20	38.19	1.67E-20
Error	15.42	64	0.24		
Total	421.99	95			

<i>Ultimate Strain</i>					
Source	Sum Sq.	DOF	Mean Sq.	F	p
Treatment	0.37	1	0.37	40.22	2.61E-08
Orientation	0.00	1	0.00	0.27	6.05E-01
Duration	0.45	7	0.07	7.00	3.42E-06
Treatment*Orientation	0.06	1	0.06	6.73	1.17E-02
Treatment*Duration	0.76	7	0.11	11.80	1.52E-09
Orientation*Duration	0.44	7	0.06	6.86	4.40E-06
Error	0.59	64	0.01		
Total	2.94	95			

<i>Elastic Modulus</i>					
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Dermis Mechanical Behaviour after Different Cell Removal Treatments

Source	Sum Sq.	DOF	Mean Sq.	F	p
Treatment	318.03	1	318.03	108.72	1.98E-15
Orientation	98.80	1	98.80	33.77	2.13E-07
Duration	748.22	7	106.89	36.54	5.10E-20
Treatment*Orientation	44.72	1	44.72	15.29	2.25E-04
Treatment*Duration	295.62	7	42.23	14.44	4.16E-11
Orientation*Duration	578.59	7	82.66	28.26	2.67E-17
Error	187.22	64	2.93		
Total	2386.48	95			

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499 **Table 2 Mechanical properties of skin in literature and in this work (average ± SD).**

Author	Skin Location (Langer Line Orientation)	UTS (MPa)	Failure Stretch	Elastic Modulus (MPa)	Reference Variables
Nì Annaidh <i>et al.</i> [8]	Middle Back (Parallel)	28.64 ± 9.03	1.46 ± 0.07	112.47 ± 36	σ_e, ϵ_e
	Bottom Back (Parallel)	17.60 ± 4.77	1.74 ± 0.32	73.81 ± 19.41	
	Middle Back (Perpendicular)	16.53 ± 5.71	1.52 ± 0.08	63.75 ± 24.59	
	Bottom Back (Perpendicular)	10.56 ± 8.41	1.61 ± 0.14	37.66 ± 36.41	
Edwards C. [33]		5-30	35-115%	15-150	Various authors
Yoder and Elliott [32]	Alloderm (Parallel)			221.48 ± 141.20	$\sigma_e, \epsilon_{Lagrange}$
	Alloderm (Perpendicular)			11.21 ± 3.53	
This work (T ₀)	Back (craniocaudal)	10.28 ± 0.96	0.77 ± 0.08	13.01 ± 2.61	$\sigma_e, \epsilon_e, E_e$
		18.38 ± 2.42			σ_{st}
		33.95 ± 4.93		43.63 ± 6.29	σ_t, E_t
	Back (medio-lateral)	7.01 ± 0.10	0.93 ± 0.15	7.20 ± 1.22	$\sigma_e, \epsilon_e, E_e$
		13.81 ± 2.80			σ_{st}
		24.11 ± 3.24		29.77 ± 7.54	σ_t, E_t

500

501 **Figure Captions**

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503 **Figure 1.** (Left) An interpolated engineering stress-strain curve, its descriptive parameters, and
504 specimen images. (Right) Experimental stress strain curves, where point ‘U’ represents the
505 average ultimate strain/stress point with its standard deviations; a) DMEM, T6, ML direction; b)
506 DMEM, T6, CC direction; c) NaOH, T6, ML direction; d) NaOH, T6, CC direction.

507 **Figure 2.** Engineering, simplified true, and true formulation curves; a) DMEM, T6, ML direction;
508 b) DMEM, T6, CC direction; c) NaOH, T6, ML direction; d) NaOH, T6, CC direction”

509 **Figure 3.** UTS values obtained from engineering, simplified true, and true formulations for
510 different decellularization treatments. Left side (grey background): results obtained along CC
511 direction; right side (white background): results obtained along ML direction

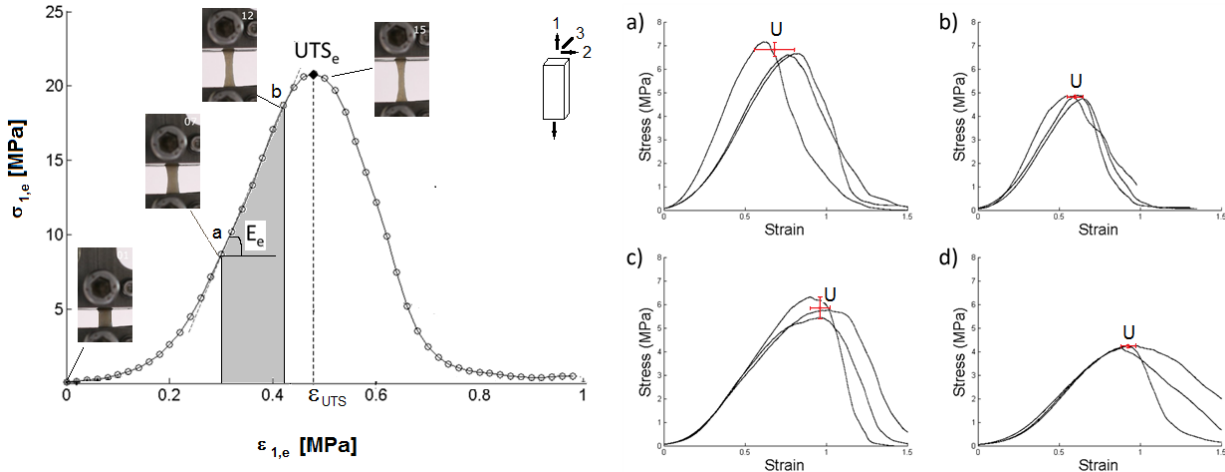
512 **Figure 4.** Engineering strain corresponding to the ultimate stress for different decellularization
513 treatments. Left side (grey background): results obtained along CC direction; right side (white
514 background): results obtained along ML direction

515 **Figure 5.** Elastic modulus values for different decellularization treatments. Left side (grey
516 background): results obtained along CC direction; right side (white background): results obtained
517 along ML direction

518 **Figure 6.** Axial stress distribution from finite element analysis: nonlinear 3D analysis (Ansys
519 Mechanical APDL); hexahedral mesh of 600 elements (SOLID186); E=14 MPa; Poisson’s ratio
520 = 0.4; all displacements have been constrained at the lower edge, while the upper edge can only
521 move vertically, where $u=2$ mm ($\epsilon=0.4$) has been applied

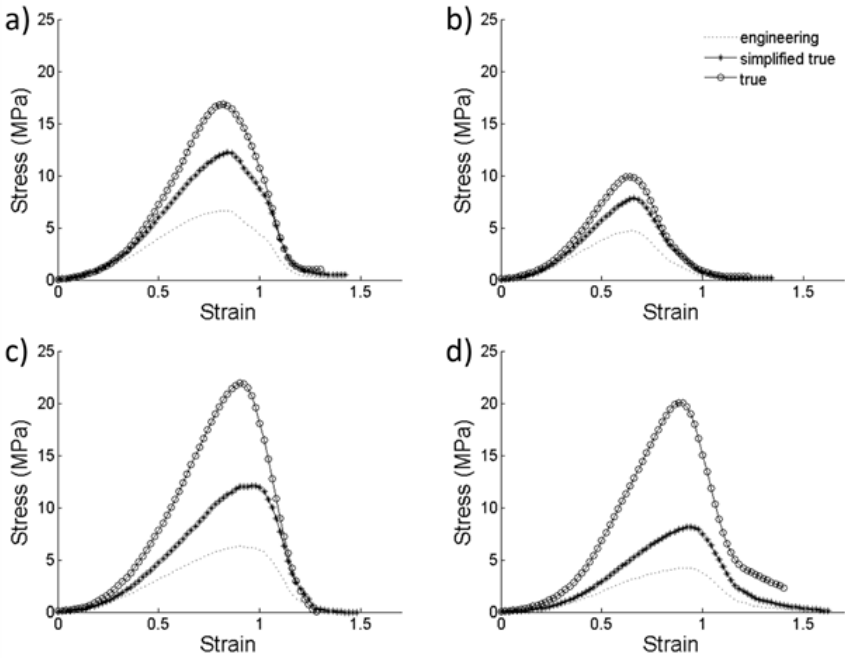
522

523 **Figure 1**



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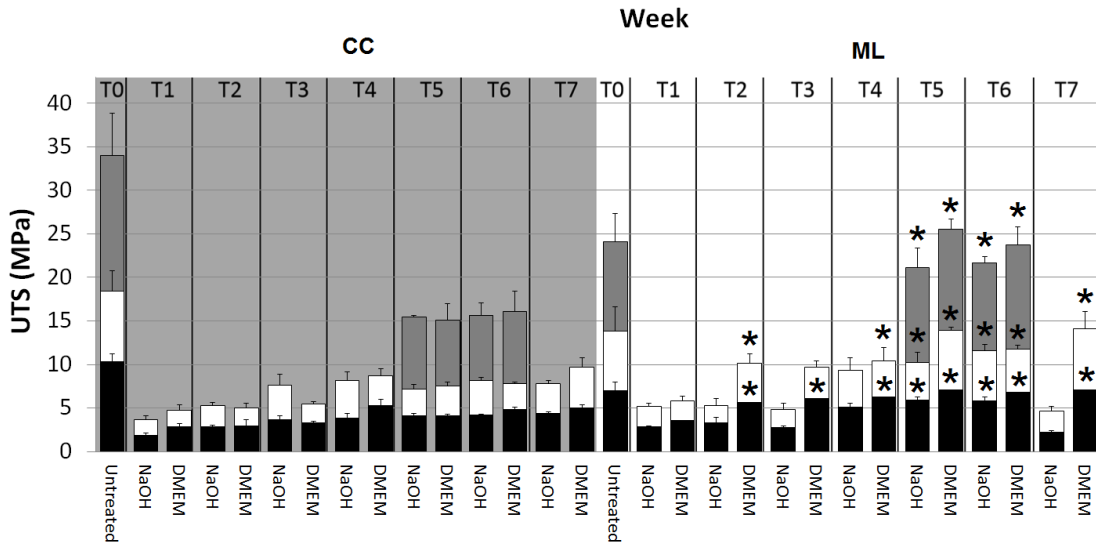
525 **Figure 2**



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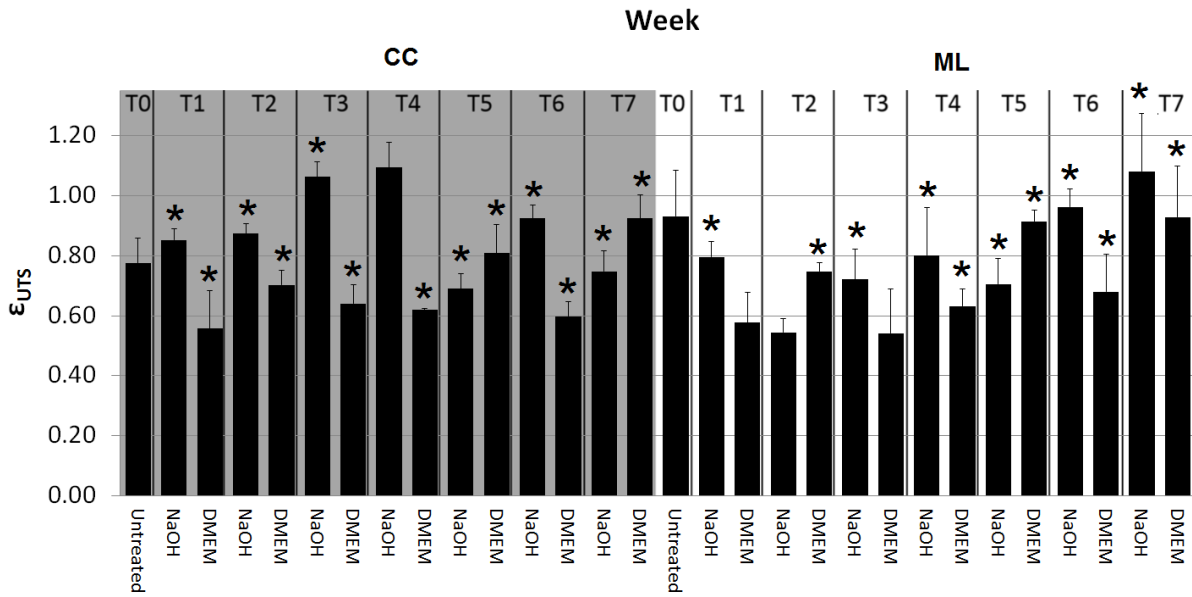
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528 **Figure 3**



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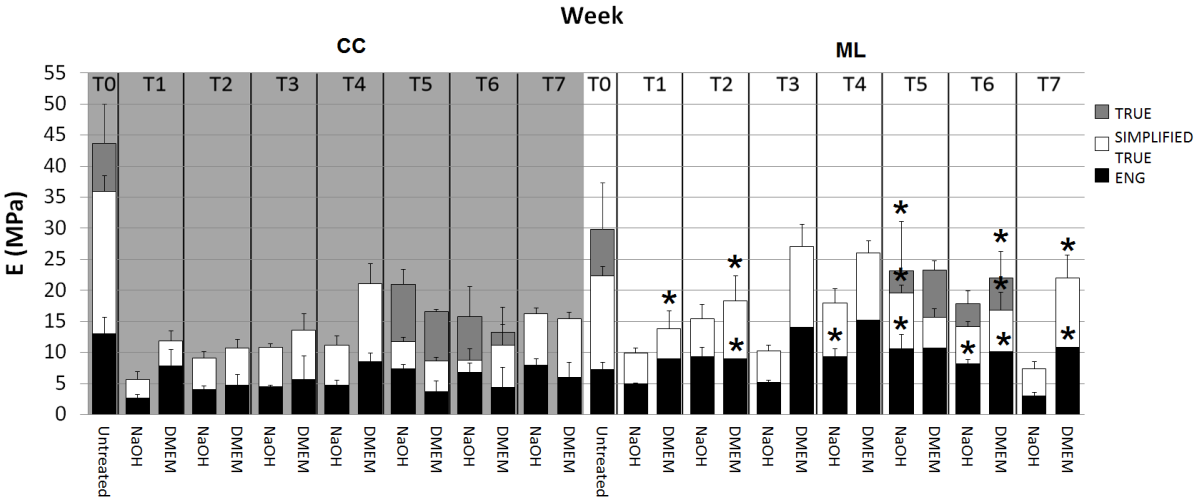
530 **Figure 4**



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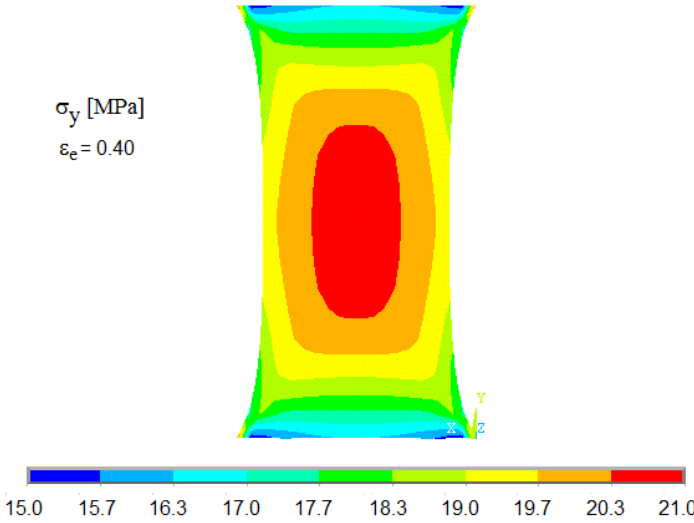
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533 **Figure 5**



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535 **Figure 6**



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