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### Long live the liver: immunohistochemical and stereological study of hepatocytes, liver sinusoidal endothelial cells, Kupffer cells and hepatic stellate cells of male and female rats throughout ageing

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1 Long live the liver: immunohistochemical and stereological study of  
2 hepatocytes, liver sinusoidal endothelial cells, Kupffer cells and hepatic  
3 stellate cells of male and female rats throughout ageing

4  
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23

24 **Abstract**

25 It has been known that both male versus female differences in liver enzyme activity and  
26 gene expression in the liver are attenuated with ageing. Nevertheless, the effect of  
27 ageing on liver structure and quantitative cell morphology remains unknown. Male and  
28 female Wistar rats of two, six, 12 and 18 months were examined. Stereological  
29 techniques and immunohistochemical tagging of Hepatocytes (HEP), liver sinusoidal  
30 endothelial cells (LSEC), Kupffer cells (KC) and hepatic stellate cells (HSC) were  
31 applied to assess the total number and number per gram of these cells throughout  
32 ageing. The mean cell volume of HEP and HSC, lobular position and liver collagen  
33 content were also evaluated with stereological techniques. The number per gram of  
34 HSC was similar for both genders and maintained throughout ageing. The mean volume  
35 of HSC was also conserved, but differences in the cell body and lobular location were  
36 observed. As to HEP, statistically significant gender differences were noted in young  
37 rats (females had smaller and more binucleated HEP) but were attenuated with ageing.  
38 The same occurred for KC and LSEC, since the higher number per gram in young  
39 females disappeared in older animals. As to liver collagen, it increased with ageing, but  
40 only in males. Herein, we highlighted that the numbers of these four cell types were  
41 related throughout ageing, with well-defined cell ratios. The shape and lobular position  
42 of HSC changed with ageing in both males and females. As to HEP, KC and LSEC, the  
43 gender dimorphism of the young rat liver disappeared with ageing.

44

## 45 **Introduction**

46 Nowadays, it is widely agreed that perfusion is reduced in the aged liver (Schmucker  
47 and Sanchez 2011; Loustaud-Ratti et al. 2016). This affects, for instance,  
48 transhepatocellular transport of dyes and IgA (Popper 1985), diffusion of small  
49 lipoproteins (Hilmer et al. 2005), bile salt formation (Le Couteur and McLean 1998;  
50 Vollmar et al. 2002) and the clearance of drugs. Differences in microsomes have been  
51 reported with ageing, with a decrease in cytochrome-P-450 concentration and NADPH-  
52 cytochrome C reductase activity (Van Bezooijen 1984; Popper 1985; Frith et al. 2009).  
53 Notably, for rodents, this occurs mainly in the male liver, because enzyme levels in  
54 females remain fairly unchanged with ageing (Kitani 1992). In effect, a sort of  
55 feminization of the male liver occurs with ageing, as enzymes more active in younger  
56 males usually decline to approach the activity levels seen in the female liver (and those  
57 less active in males increase up to the female levels) (Kitani 1992; 2007). Curiously,  
58 this pattern also appears to exist at the level of gene expression (Kwekel et al. 2010).  
59 Nevertheless, ageing effects on liver structure are much less clear. The state of the art in  
60 this field is characterised by few consistent observations and a lack of correlation  
61 between structural and functional data (Zeeh 2001). Furthermore, to the best of our  
62 knowledge, the liver structure of the aged male and female liver has never been studied  
63 in detail by quantitative morphology.

64 Despite this, the last decade has shed some light on liver ageing. It has been reported  
65 that sinusoids in the aged liver have fewer *fenestrae*, surrounded by basal lamina and  
66 collagen, leading to so-called pseudocapillarisation (Le Couteur et al. 2001; 2008).  
67 Furthermore, intralobular collagen was reported to increase with ageing (Gagliano et al.  
68 2002). Since this collagen is mainly produced by hepatic stellate cells (HSC), at least in  
69 pathological conditions, it could be hypothesized that morphological differences would  
70 appear with ageing. Only a few studies on liver ageing have explored the HSC role on  
71 liver ageing, either in a qualitative (Enzan et al. 1991) or in a semi-quantitative  
72 perspective (Martin et al. 1992; Imai et al. 2000; Vollmar et al. 2002; Warren et al.  
73 2011) and their conclusions were controversial. Moreover, studying gender ageing  
74 differences among rats is quite relevant due to the common practice of using males and  
75 females interchangeably for experimental studies, as well as *in vitro* protocols. For  
76 instance, older rats — typically male and/or retired female breeders (Ramadori and  
77 Saille 2002; Tacke and Weiskirchen 2005; Friedman 2008) — are recommended for use

78 when isolating HSC; however, it is unknown whether HSC differ in quantitative  
79 morphology between genders.

80 In order to reveal eventual ageing differences a quantitative approach should be applied  
81 (e.g. using stereology), since the qualitative morphology alone may overlook important  
82 structural changes. Other methods, such as cell isolation, are unable to reveal liver cell  
83 ratios, since the cell yield from these methods varies between parenchymal and non-  
84 parenchymal cells. Nevertheless, an in-depth knowledge of liver structure (and cell  
85 ratios) is important for the bioengineering construction of artificial livers and for *in vitro*  
86 studies, since precise cell ratios are necessary to model paracrine effector mechanisms  
87 in co-culture models. Studies *in vitro* have used ratios of parenchymal and non-  
88 parenchymal cells varying from 10:1 to 1:10 have been used (Bathia et al. 1999), which  
89 may not mirror the *in vivo* organization of the aged liver. Furthermore, it has been  
90 stressed that the true aspects of ageing are difficult to ascertain from a simple  
91 comparison between young and old animals (since changes can occur in between), or  
92 from the study of only the male gender (Kitani 1992; Schmucker 2001).

93 In this study we used design-based stereology methods to study morphological changes  
94 of the rat liver throughout ageing in both males and females. We looked for differences  
95 in the size of lobules, collagen content and in the total number (N) and number per gram  
96 (N/g) of hepatocytes (HEP), Kupffer cells (KC), HSC and liver sinusoidal endothelial  
97 cells (LSEC). Finally, we studied the cell volume as well as the position of HSC in liver  
98 lobules.

99

## 100 **Materials and Methods**

### 101 ***Animals***

102 We used male and female Wistar rats (n = 5 per group) of two months old, adult (six  
103 months old), middle-aged (12 months old) and old (18 months old), initially bought  
104 from Charles-River Laboratories (Barcelona, Spain). When considering the mean  
105 lifespan of this strain, these ages correspond to around 10%, 25%, 50% and 75% of  
106 their lifespan respectively (Porta et al. 1980; Sawada and Carlson 1987, Manikonda and  
107 Jagota 2012). All the animals had been weaned at 20 days and were kept in standard  
108 conditions, receiving water and food (Mucedola® 4RF21, Settimo Milanese, Italy) *ad-*  
109 *libitum*. The rats were housed in pairs or individually (old males) in a controlled  
110 environment [temperature (25°C) and 12 hours light-dark cycle]. Animal management  
111 followed European Union Directives (1999/575/CE and 2010/63/UE) for the protection

112 of animals used for scientific purposes and the study was approved by local ethical  
113 authorities (ORBEA ICBAS-UP Project 152/2016).

#### 114 *Tissue Preparation*

115 Sampling was performed during the morning period (from 10:00 to 12:00), to  
116 circumvent oscillations in liver function due to circadian rhythmicity (Davidson et al.  
117 2004). In females, daily vaginal cytologies were observed, in order to avoid collecting  
118 samples in proestrous/oestrous days. Beforehand, animals were deeply anaesthetised  
119 with ketamine plus xylazine. Blood was collected and centrifuged to obtain serum for  
120 assessing alanine transaminase and aspartate transaminase levels. Transcardiac  
121 perfusion was performed with an isosmotic solution; the liver was weighed and its  
122 volume determined by the Scherle's method, as detailed elsewhere (Marcos et al. 2012).  
123 A smooth fractionator sampling scheme was applied (Marcos et al. 2012): half of the  
124 paraffin blocks was used for thick sections (30  $\mu\text{m}$  thick) and exhaustively sectioned,  
125 whilst the other half was used for thin sections (3  $\mu\text{m}$  thick). Of the thick sections, five  
126 sections in every 30 were sampled in order to immunostain against: 1) glial fibrillary  
127 acidic protein for estimating the N and N/g of HSC; 2) ED2 for estimating the N and  
128 N/g of KC; 3) E-cadherin, to differentiate mononucleate HEP from binucleated HEP,  
129 estimating their percentage, and to assess the N and N/g of HEP; 4) Von Willebrand  
130 Factor to estimate the N and N/g of LSEC; 5) glial fibrillary acidic protein and  
131 glutamine synthetase [an established marker of centrilobular HEP (Gebhardt and  
132 Mecke 1983)] to evaluate the lobular distribution of HSC. Thin sections were used for  
133 immunostaining against: 1)  $\alpha$ -smooth muscle actin to evaluate the existence of activated  
134 HSC; 2) glial fibrillary acidic protein to determine the relative volume of HSC (whole  
135 cell); 3) glutamine synthetase, to estimate the lobular size, by measuring the porto-  
136 central distance. Thin sections were also used for assessing liver collagen, by Sirius red  
137 staining. In addition, tiny liver fragments ( $< 0.5 \text{ mm}^3$ ) were removed from the rat liver  
138 for electron microscopy. These were fixed in 2.5% glutaraldehyde in 0.1M phosphate  
139 buffer (pH = 7.4) for two hours and subsequently post-fixed in phosphate buffered 1%  
140  $\text{OsO}_4$  for another two hours. After dehydration in ethanol and propylene oxide, the  
141 pieces were embedded in epoxy resin. Semithin sections were obtained and stained with  
142 methylene blue-azur II, which were used to quantify volume densities of HSC (cell  
143 body). Additionally, ultrathin sections were obtained, contrasted with uranyl acetate and  
144 lead citrate and observed in a transmission electron microscope, JEOL 100CXII, at 60  
145 kV.

146

147

148

### 149 ***Immunohistochemistry***

150 The protocol used for thick sections has been previously described (Marcos et al. 2004;  
151 2006; Santos et al. 2009). Briefly, antigen recovery was carried out in microwave [four  
152 plus four minutes, at 600 W, in buffered citrate 0.01M (pH = 6.0)] and a streptavidin–  
153 biotin protocol was used (Histostain Plus, Invitrogen, Camarillo, California) for all  
154 antibodies, except for LSEC [pre-treatment of tissues with pepsin (Sigma, St. Luis,  
155 Missouri) 0.4% in HCl 0.01M for 30 minutes]. For glial fibrillary acidic protein, we  
156 used 1:3000 rabbit polyclonal antibody (Dako, Glostrup, Denmark), whereas for ED2  
157 and E-cadherin we used monoclonal mouse antibodies, from Serotec (Oxford, United  
158 Kingdom) diluted at 1:100 and from Dako (clone NCH 38) diluted at 1:250,  
159 respectively. It is opportune to mention that ED2 is unanimously recognized as a marker  
160 of fully differentiated, long-lived KC (Roskams et al. 2007; Santos et al. 2009).  
161 Additionally, we used an antibody against Von Willebrand Factor from Dako, diluted at  
162 1:3200, in order to tag LSEC. All the slides were incubated for four days at 4° C.

163 For the double immunohistochemistry, slides were also placed in the microwave (this  
164 time for three cycles of four minutes at 600W in buffered citrate). After blocking  
165 endogenous biotin and peroxidase, the first streptavidin–biotin protocol was followed  
166 with antibody against glial fibrillary acidic protein (1:1500 dilution for four days at  
167 4°C). Slides were developed for two minutes in 0.05% 3,3'-diaminobenzidine (Dako) in  
168 Tris-buffered saline with 0.03% H<sub>2</sub>O<sub>2</sub>. Sections were then rinsed in tap-water and  
169 dipped in 50 mM glycine buffer (pH = 2.2) for five minutes, to strip off the antibodies  
170 of the first immunoreaction. The second streptavidin–biotin protocol against glutamine  
171 synthetase followed, using 1:4000 rabbit polyclonal antibody (graciously provided by  
172 Professor Rolf Gebhardt, University of Leipzig), for another four days at 4°C. Slides  
173 were developed with aminoethylcarbazole (Dako) for 10 to 20 minutes (final red colour  
174 controlled by microscopic observation) and mounted in Aquatex (Dako).

175 To quantify glial fibrillary acidic protein in thin sections, a streptavidin–biotin protocol  
176 was also used (Histostain Plus), but with lower dilutions (1:1200) and overnight  
177 incubation. The blocking solution, the secondary antibody and the streptavidin–  
178 peroxidase complex were all applied for 20 minutes and colour development in  
179 diaminobenzidine was restricted to two minutes. As for  $\alpha$ -smooth muscle actin

180 immunostaining, slides were placed in a pressure cooker for three minutes in citrate  
181 buffer (pH = 6.0). After rinsing in phosphate buffered saline, a polymer based  
182 immunohistochemical protocol was followed [Novocastra Novolink Polymer (Leica  
183 Biosystems, Newcastle, United Kingdom)]. The protein blocking solution was applied  
184 for five minutes and  $\alpha$ -smooth muscle actin antibody (clone HM45, Dako) diluted at  
185 1:500 immersed the slides overnight. The post-primary solution and polymer were both  
186 applied for 30 minutes. Finally, slides were developed for two minutes in  
187 diaminobenzidine. For assessing the porto-central distance, immunohistochemistry  
188 against glutamine synthetase was used (diluted at 1:4000), following the protocol for  
189 glial fibrillary acidic protein immunostaining in thin sections.  
190 Positive and negative controls (omission of first antibody and replacement by non-  
191 immune serum) were included, both in thin and thick sections, and all slides were  
192 evaluated blindly (*i.e.*, the observer was unaware of the gender or age of the animal), to  
193 avoid eventual observer-related bias.

194

#### 195 ***Histochemistry with Sirius-red***

196 Five thin sections were randomly selected per animal, de-waxed and hydrated. The  
197 counterstaining was achieved with celestial blue and haematoxylin, each for 5 minutes.  
198 After washing in tap water, the Sirius red (Sigma, coloration index 35782) dissolved in  
199 picric acid (1 mg/ml) was applied for 1 hour at room temperature (Kumar 2005). After  
200 washing in acidified water (1% acetic acid), the sections were dehydrated in ethanol,  
201 cleared in xylene and mounted in DPX.

202

#### 203 ***Morphometrical Analysis***

204 In the thin sections, the linear distance between a central venule (surrounded by HEP  
205 immunostained against glutamine synthetase) and the closest portal tract was measured  
206 using the CAST-Grid software (version 1.5, Olympus). Both peripheral (closer to  
207 Glisson's capsule) and inner lobules were considered for the measurements.

208

#### 209 ***Stereological Analysis***

210 A stereology workstation with CAST-Grid software was used (Marcos et al. 2012). At  
211 the monitor, a final magnification of 4750x allowed easy and accurate recognition of all  
212 cells. Throughout the disector height (20  $\mu\text{m}$ ), a software generated counting frame with  
213 defined areas (1673  $\mu\text{m}^2$ , 1267  $\mu\text{m}^2$  and 418  $\mu\text{m}^2$  for HSC, KC/LSEC and HEP,



214 respectively) was used for unbiased counting. For assessing the position of HSC in liver  
215 lobules, a systematic uniform random sampling was applied, but HSC were counted  
216 only if fields were in the vicinity of the portal tracts or central venules (5-6 HEP around  
217 those landmarks were settled upon as boundaries). For the purpose of counting cells,  
218 their nucleus was considered the counting unit (in the case of binucleated HEP, this was  
219 predetermined to be the first nucleus in focus), and cells were counted following optical  
220 disector rules (Marcos et al. 2012). The N of cells in the whole liver was estimated  
221 using optical fractionator formulae (Marcos et al. 2004; 2012). Simultaneously, the N/g  
222 was determined, as it is useful for comparing animals with different liver weights. The  
223 coefficient of error of the number of cells counted was also estimated (Marcos et al.  
224 2004; 2012).

225 Additionally, the mean cell volume, so-called number-weighted mean cell volume, of  
226 mononucleate and binucleated HEP was estimated by the nucleator method (Gundersen  
227 1988; Marcos et al. 2012). In the latter, the first nucleus with nucleolus in focus was  
228 considered for the measurements (Marcos et al. 2012). In the case of HEP with two  
229 nucleoli (or more), two (or more) measurements were performed (Gundersen 1988).

230 Semithin and glial fibrillary acidic protein immunostained sections were used,  
231 respectively, in order to estimate the relative volume of the cell body and whole cell of  
232 HSC. In both cases, point counting (grid with 108 points) was used to estimate the  
233 relative volume (Figure 1). The number-weighted mean volume was then obtained by  
234 an indirect approach, through the division of the relative volume by the relative number  
235 of HSC per unit of volume (corrected for paraffin shrinkage) (Marcos et al. 2012). As to  
236 the assessment of collagen content, point-counting (grid with 36 points) was also  
237 performed in the CAST-Grid software. In a preliminary study, we have shown that this  
238 test-system allowed an easy discrimination of collagen fibres (Marcos et al. 2015).

239

#### 240 *Statistical analysis*

241 The normality of the data was checked using the Shapiro-Wilk's test. Pearson's  
242 correlation analysis was performed to detect linear correlations. After checking the  
243 homogeneity of variances (Levene's test) a two-way ANOVA was performed taking  
244 into consideration the effects of gender and ageing. When significant differences  
245 existed, multiple comparisons were done using the post-hoc Tukey's test. Statistical  
246 significance level was set at  $p \leq 0.05$ . The software SPSS 18 (IBM, Armonk, United

247 States of America) was used. Quantitative results are presented with their mean and  
248 standard deviation.

249

250

251

## 252 **Results**

### 253 *Qualitative findings*

254 All livers displayed a normal morphology, without noticeable differences across  
255 animals at optical and electron microscopy. A consistent and reliable marking of HEP  
256 was achieved with E-cadherin, allowing a clear distinction between mononucleate and  
257 binucleated HEP. For glial fibrillary acidic protein, HSC were immunostained in both  
258 periportal and centrilobular areas (Supplemental figure 1). The HSC of aged rats had  
259 larger and more numerous lipid droplets than young rats; these cells occasionally  
260 protruded into sinusoids (Figure 1). However, no differences were noticeable between  
261 HSC of males and females. No staining of HSC with  $\alpha$ -smooth muscle actin antibody  
262 was observed (Supplemental figure 2). Fully differentiated KC also exhibited a stellate  
263 appearance with ED2, but with shorter and much thicker cytoplasmic processes.  
264 Regarding LSEC, they had a characteristic dark nucleus layered on a thin rim of  
265 immunomarking against Von Willebrand Factor (Figure 2).

266

### 267 *Quantitative findings*

268 The livers of males were heavier ( $p < 0.001$ ) than those of females ( $15.15 \pm 1.6$  g *versus*  
269  $9.62 \pm 1.2$  g, respectively). The liver-to-body weight ratio was highest at the age of two  
270 months ( $4.27\% \pm 0.7\%$ ,  $p < 0.01$ ) compared to other age groups ( $2.72\% \pm 0.3\%$ ); no  
271 gender differences were observed. A strong correlation was observed between liver and  
272 body weight ( $r = 0.77$ ,  $p < 0.001$ ). Hepatic transaminases values were within the  
273 reference ranges, presenting no statistically significant differences ( $43.1 \pm 4.0$  IU/L and  
274  $39.1 \pm 9.3$  IU/L for alanine transaminase, and  $101.4 \pm 17.6$  IU/L and  $105.2 \pm 14.5$  IU/L  
275 for aspartate transaminase in males and females, respectively).

276 An average of 62 lobules per rat was analysed by morphometry. The average porto-  
277 central distance was  $450 \pm 17$   $\mu$ m in males and  $412 \pm 22$   $\mu$ m in females (data corrected  
278 for paraffin shrinkage). No statistically significant differences were observed for the  
279 size of lobules throughout ageing and between genders (Table 1).

280 An average of 216 fields per animal was screened to assess liver collagen. The amount  
281 of intralobular collagen corresponded to 56% in males and 46% in females, without  
282 significant differences with ageing. This collagen was moderately correlated with the N  
283 of HSC ( $r = 0.50$ ;  $p < 0.01$ ) and with the N of HEP ( $r = 0.47$ ;  $p < 0.01$ ). Liver collagen  
284 was influenced by gender ( $p < 0.001$ ) and ageing ( $p < 0.01$ ), namely in males. In fact,  
285 collagen was more abundant in males than in females in adult and old rats (Figure 3).  
286 This was due to intralobular collagen, since the collagen around central veins and in  
287 portal tracts was maintained throughout ageing and gender (varying between 0.9 and  
288 1.3% of liver collagen).

289 From each rat, between 291 and 780 optical disectors were analysed to obtain the N and  
290 N/g of HSC, HEP, KC and LSEC; and between 150 and 216 fields per rat were assessed  
291 to determine the relative volume of HSC (cell body and whole cell). The N of HSC was  
292 higher in males ( $209 \pm 14 \times 10^6$ ) than in females ( $154 \pm 15 \times 10^6$ ) ( $p = 0.016$ ), but the  
293 N/g was similar across genders ( $\approx 14.6 \pm 1.3 \times 10^6$ ) (Figure 4). Neither of these  
294 parameters was associated with ageing. A correlation was observed between the N of  
295 HSC and *i*) liver weight ( $r = 0.85$ ,  $p < 0.01$ ), *ii*) N of HEP ( $r = 0.73$ ;  $p < 0.0001$ ) and *iii*)  
296 with the N of KC ( $r = 0.53$ ,  $p < 0.01$ ). Due to the increased volume of lipid droplets  
297 present in older HSC, the number-weighted mean volume of the cell body increased  
298 with ageing ( $144 \pm 61 \mu\text{m}^3$  to  $576 \pm 104 \mu\text{m}^3$ ) ( $p < 0.01$ ), but the volume of the whole  
299 cell was relatively stable, varying from  $593 \pm 134 \mu\text{m}^3$  to  $796 \pm 192 \mu\text{m}^3$  (Figure 5).  
300 The distribution of HSC was not influenced by gender; however, an ageing pattern was  
301 observed ( $p < 0.001$ ): in younger animals HSC were more frequently located  
302 pericentrally ( $56.5 \pm 4.9\%$ ) whereas in older animals these cells were more abundant in  
303 a periportal location ( $61.2 \pm 6.7\%$ ).

304 The N of HEP did not vary with ageing ( $\approx 2.0 \pm 0.3 \times 10^9$ ), in contrast with the N/g of  
305 HEP ( $159 \pm 33 \times 10^6$  in young and  $184 \pm 26 \times 10^6$  in old) ( $p < 0.05$ ) (Figure 6). The N  
306 of HEP was statistically correlated with *i*) body and *ii*) liver weights ( $r = 0.60$ ;  $p <$   
307  $0.0001$ , for both) and *iii*) with the N of KC ( $r = 0.5$ ,  $p < 0.01$ ), whereas the N/g of HEP  
308 was correlated with the N/g of KC and N/g of binucleated HEP ( $r = 0.94$ ,  $p < 0.001$  and  
309  $r = 0.75$ ,  $p = 0.02$ , respectively). Gender differences were observed in the N/g of HEP,  
310 but these were restricted to younger animals ( $136 \pm 11 \times 10^6$  in males and  $183 \pm 39 \times$   
311  $10^6$  in females) ( $p = 0.016$ ). Similarly, gender differences existed for binucleated HEP,  
312 as well as their percentage, which were higher in females ( $25 \pm 4\%$  in young males and  
313  $34 \pm 5\%$  in young females), but these differences were attenuated with ageing ( $27 \pm 5\%$

314 and  $31 \pm 4\%$  in old males and females, respectively) (Figure 6). The percentage of  
315 binucleated HEP was negatively correlated with the body weight ( $r = -0.81$ ,  $p = 0.015$ ).  
316 For the number-weighted mean cell volume no statistically significant difference was  
317 observed with ageing and gender [except between mononuclear HEP in young males  
318 and females ( $5861 \pm 369 \mu\text{m}^3$  and  $4915 \pm 293 \mu\text{m}^3$ , respectively) ( $p < 0.01$ )] (Figure 7).  
319 In average, the volume of binucleated HEP was  $7177 \pm 752 \mu\text{m}^3$ , being 31 to 59%  
320 greater than that of mononucleate HEP ( $p < 0.001$ ).

321 The N of fully differentiated KC was moderately correlated with liver weight ( $r = 0.67$ ,  
322  $p < 0.001$ ). Overall, this parameter was stable with ageing ( $\approx 286 \pm 58 \times 10^6$ ), as was the  
323 N/g of KC ( $\approx 23 \pm 3 \times 10^6$ ); however, gender differences were observed in young  
324 animals ( $19 \pm 3 \times 10^6$  and  $30 \pm 6 \times 10^6$  in males and females, respectively) ( $p = 0.016$ )  
325 (Figure 8). Likewise, the N of LSEC was also stable with ageing and gender ( $\approx 802 \pm$   
326  $25 \times 10^6$ ). Nevertheless, gender differences existed for the N/g ( $p < 0.001$ ) and these  
327 occurred mostly in young animals ( $40 \pm 4 \times 10^6$  and  $95 \pm 11 \times 10^6$  in males and females,  
328 respectively) ( $p < 0.0001$ ) (Figure 9).

329 By estimating the N of HEP, HSC, KC and LSEC in the same set of animals, it was  
330 possible to estimate the ratios among these cell types in the male and female rat liver  
331 (Figure 10). Overall, the percentage of HEP, LSEC, KC and HSC in the rat liver was  
332 60%, 21%, 8.9% and 5.8%, respectively in males, and 56%, 25%, 7.8% and 4.8%,  
333 respectively in females.

334 It is noteworthy that the coefficient of error for estimations of cell number (N) were  
335 always below the recommended threshold of 10% (Marcos et al. 2012), being  
336 comprised of between 3.9% and 6.8%. Therefore, the sampling procedure was  
337 responsible for up to 24% of the total observed variance — *i.e.*, the variance due to the  
338 methodological procedures was much less important than the biological variability.

339

## 340 **Discussion**

341 To the best of our knowledge, this is the first study of liver ageing using quantitative  
342 morphology that addressed differences in both males and females. Wistar rats were used  
343 in this study because, on one hand, this is one of the most common stock of animals  
344 used in liver research, and on the other hand, this strain has few age-related liver lesions  
345 — the only lesion consistently reported are altered cell foci, which tend to occur at a  
346 later age, around 2.5 years (Van Bezooijen 1984). Wistar rats therefore differ from other

347 strains used in gerontology research, such as Fischer or Sprague-Dawley rats. Fischer  
348 strains have altered cell foci at an earlier age than Wistar, and may bare focal chronic  
349 hepatitis and bile duct hyperplasia (Van Bezooijen 1984). Sprague-Dawley rats have  
350 been described as exhibiting periportal inflammation, sinusoidal enlargement, fatty  
351 change and eosinophilic foci on reaching older ages (Van Bezooijen 1984; Sakai et al.  
352 1997). As to sporadic tumors, aged Wistar rats have an increased incidence of pituitary  
353 adeno(carcino)ma, mammary adeno(carcino)ma (in females) and Leydig cell tumor (in  
354 males), but hepatocellular neoplasms are reported to be rare (Eiben and Bomhard 1999).  
355 The major drawback of using the Wistar strain on liver ageing studies, however, is that  
356 animals have to be aged in-house, since suppliers have a limited number of available  
357 rats (females over six months cannot be bought from existing companies).

358 Overall, this study highlighted statistically significant correlations between the numbers  
359 of different liver cell types in the rat liver. This is a new finding that supports the  
360 existence of a morphofunctional organisation, with well-defined ratios of parenchymal  
361 and non-parenchymal cells maintained throughout ageing (Figure 10). These ratios have  
362 been previously hypothesized by Rojkind et al. (2011) but have never been determined  
363 throughout ageing in males and females. Considering the volume estimation of  
364 mononucleate and binucleated HEP and their relative abundance, a theoretical porto-  
365 central cell cord composed of  $17 \pm 1$  HEP in males and  $16 \pm 1$  HEP in females was  
366 estimated. Another major conclusion that can be drawn from this study is that the  
367 ageing process attenuated most of the cytological differences in liver cells. The gender  
368 dimorphism that existed in young animals for HEP, KC and LSEC, but not for HSC,  
369 disappeared at older ages. Such a trend has never been revealed by morphology, but it  
370 actually follows known patterns for enzymatic activities (Kitani 2007) and gene  
371 expression (Kwekel et al. 2010). An exhaustive micro-array gene expression study with  
372 rats highlighted that most gene differences decreased by middle-age and were  
373 completely abolished in old animals (Kwekel et al. 2010).

374 Our initial hypothesis that morphological differences for HSC could exist with ageing  
375 was not supported by our data, since no differences were observed in the number or  
376 mean volume of HSC. So far only HSC “numbers per area” have been determined along  
377 ageing, however the studies reached to controversial conclusions. According to Cogger  
378 and Le Couteur (2009) and Warren et al. (2011), HSC doubled their number with  
379 ageing. Whereas Enzan et al. (1991) reported no significant differences and Vollmar et  
380 al. (2002) suggested that the number of HSC could decrease up to 30% with ageing.

381 However, it should be acknowledged that two-dimensional counts (“numbers per area”)  
382 are biased and may not reveal the three-dimensional reality of biological tissues  
383 (Mandarim-Lacerda 2003; Marcos et al. 2012). The larger cell body of an aged HSC  
384 could be prone to an overestimation bias in two-dimensional counts. The shift from a  
385 centrilobular to a periportal predominance of the HSC with ageing is another new  
386 finding. It has been reported that quiescent HSC are able to slowly move through the  
387 space of Disse (Senoo et al. 2007; Friedman 2008), being dynamic in the changeable 3D  
388 structure of the sinusoids (Sato et al. 2003; Senoo et al. 2007). It has long been known  
389 that the matrix composition differs along the porto-central axis (Reid et al. 1992;  
390 Roskams et al. 2007; Senoo et al. 2010). It is conceivable that an increase of factors that  
391 drive HSC migration, *e.g.* transforming growth factor- $\beta$ 1 (Yang et al. 2003), could  
392 occur with ageing. It is noteworthy that aged HSC were not activated, being  $\alpha$ -smooth  
393 muscle actin antibody negative, as previously described (Warren et al. 2011). However,  
394 their shape was different with ageing. Classical and more recent studies have reported  
395 that HSC in aged animals appear swollen, having significantly more and larger lipid  
396 droplets than in young animals (Enzan et al. 1991; Warren et al. 2011). This was  
397 confirmed by us, since a significant increase in the number-weighted mean volume of  
398 the cell body occurred with ageing. Based on such differences, some authors concluded  
399 that aged HSC are larger than young HSC (Vollmar et al. 2002; Schmucker 2005);  
400 however our study suggests that, based on the number-weighted mean volume of HSC,  
401 the cells actually change from a small cell body with long and thin extensions in  
402 youngsters to a large cell body with thicker and much shorter extensions in older rats.  
403 Actually, this large cell body justifies the recommendation of using old animals for  
404 isolating HSC (Ramadori and Saille 2002; Tacke and Weiskirchen 2005; Friedman  
405 2008) — since cells contain more lipid droplets they can be better separated by gradient  
406 centrifugation. Moreover, the larger cell body of HSC should also contribute to the  
407 blood flow reduction in sinusoids of older animals (Vollmar et al. 2002; Warren et al.  
408 2011) because it protrudes into sinusoids — as has previously been reported (Warren et  
409 al. 2011) — and HSC shift to periportal areas. It may be hypothesized that the shorter  
410 processes of aged HSC should surround and control the blood flow of fewer sinusoids  
411 than in youngsters, in whom HSC encircle more than two sinusoids (Friedman 2008).  
412 Whether or not hyperplasia and/or hypertrophy of HEP occur with ageing remain  
413 controversial questions. The porto-central distance estimated in this study is in

414 accordance with previous reports for rats (Wagenaar et al. 1994; Ruijter et al. 2004;  
415 Warren et al. 2008) and no ageing differences in this axis were found. In the ageing  
416 process it is thought that only enlargement of previously existing lobules occurs  
417 (Vollmar et al. 2002). However, considering that the N/g of HEP increases by 35%  
418 between young and old males and the porto-central axis is maintained, two hypotheses  
419 may be placed: lobules grow in height and/or new lobules could be formed. Taking into  
420 account that the relative volume of collagen within portal tracts and central venules was  
421 maintained throughout ageing, an increase in height (*i.e.*, taller lobules) would probably  
422 be more important. This increased height, bearing an increased number of primary  
423 classical lobules at the base (liver surface), would justify the greater number of  
424 superficial lobules reported in the rat (Papp et al. 2009).

425 Another controversy surrounding HEP relates to the increase of binucleated HEP with  
426 ageing (Wheatley 1972, Schmucker 1998). It is unanimously agreed that polyploidy  
427 increases in the aged liver and some authors also reported an upsurge in binuclearity  
428 (Popper 1985; Schmucker 1998; Malarkey et al. 2005). Even so, in our set of rats, we  
429 did not find statistically significant ageing differences in the N of binucleated HEP or in  
430 their percentage. This is in agreement with previous studies, based on different  
431 methodologies in mouse and rat (Epstein 1967; Wheatley 1972; Faggioli et al. 2011). It  
432 is noteworthy that the percentage of binucleated HEP was negatively correlated with  
433 body and liver weight. This has already been reported in the rat elsewhere (Vinogradov  
434 et al. 2001), with very similar figures ( $r \approx -0.57$ ).

435 The N of fully differentiated KC has never been evaluated throughout ageing, to the  
436 best of our knowledge. Hilmer et al. (2007) reported a 3-fold increase in “numbers per  
437 area” of KC with ageing, using thin paraffin sections stained by haematoxylin-eosin.  
438 Technical concerns may explain the differences to our study, such as the use of  
439 haematoxylin-eosin, which is undesirable for quantification due to uncontrolled bias in  
440 the counts, but also size differences (larger KC in old animals) may lead to  
441 overestimations when using “numbers per area”. Other studies have suggested that the  
442 volume of KC increases with ageing (Martin et al. 1992; 1994), probably due to an  
443 accumulation of non-functional material in cytoplasm (Martin et al. 1994).

444 Classical and more recent studies have addressed the structure of LSEC throughout  
445 ageing. While the reduction in the number of *fenestrae* (ageing defenestration) in LSEC  
446 is nowadays well documented in different species (Le Couteur et al. 2001; 2008;  
447 Cogger and Le Couteur 2009), the N and N/g have been sparsely detailed. Still, it was

448 proposed that the number and percentage of LSEC were constant throughout ageing (De  
449 Leeuw et al. 1990), and it was reported that numbers of sinusoids remained fairly  
450 unchanged during the lifespan of rats (Vollmar et al. 2002), which is consistent with our  
451 findings. It has been determined that N/g of LSEC was around  $20 \times 10^6$  (De Leeuw et  
452 al. 1990) and that these cells comprise 20% of all liver cells (Malarkey et al. 2005).  
453 According to our findings, the N/g of LSEC is higher ( $49 \times 10^6$  and  $79 \times 10^6$  in males  
454 and females respectively) and these cells comprise 21% to 26% of all liver cells.

455 The liver has much less collagen than most other organs — only  $\approx 5\%$  (Roskams et al.  
456 2007; Friedman 2008), as we observed herein. Previous studies have followed the  
457 collagen content throughout ageing, but only in males, and noted that it increased with  
458 ageing (Porta et al. 1981; Gagliano et al. 2002). Curiously, both studies documented a  
459 collagen peak in 6 months' animals, as we observed. The reason for such an increase in  
460 collagen is unknown, but it may traduce a remodelling activity with formation of new  
461 lobules, which is believed to occur during liver growth (Roskams et al. 2007). At that  
462 age, as well as in older animals, it has been shown that the metalloproteinase activity  
463 was significantly reduced (Gagliano et al. 2002). It should be emphasised that  
464 (intralobular) collagen cannot be solely related to HSC, since the expression of collagen  
465 I has been attributed to LSEC, HSC and HEP (Roskams et al. 2007). In this vein, we  
466 observed a significant correlation between collagen content and two latter cell types,  
467 suggesting that both HSC and HEP may be important for collagen synthesis in the  
468 normal organ.

469 In conclusion, this study contributes to the state of the art about the process of liver  
470 ageing, highlighting that the liver structure is well preserved. Herein, we provided  
471 defined ratios between cells, which are relevant for bioengineering construction of  
472 artificial livers and for *in vitro* studies, namely using co-culture models. Except for  
473 collagen content, the male/female differences are attenuated by ageing and, in this vein,  
474 no major cytological or structural changes in normal liver cells should be able to  
475 compromise the long life of the liver.

476

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639

640 Figure 1 – Semithin section of liver parenchyma of an old rat. A hepatic stellate cell  
641 (arrows), engorged with lipid droplets, protrudes into a sinusoid. For illustrative  
642 purposes, the counting grid (composed of 108 points) used for estimating the relative  
643 volume of the cell body of hepatic stellate cells is shown; in this case, two points (red)  
644 would be counted.

645 Figure 2 – Thick liver sections (30  $\mu\text{m}$ ) immunostained against: (A) E-cadherin to  
646 highlight the borders of mono- and binucleated hepatocytes; (B) ED-2 to tag fully  
647 differentiated Kupffer cells; (C) Von Willebrand factor to depict liver sinusoidal  
648 endothelial cells and (D) glial fibrillary acidic protein to mark hepatic stellate cells. The  
649 counting grid is shown for illustrative purposes, being larger (D) for less abundant cells  
650 and smaller for the most numerous (A). Cells were counted if their nucleus was inside  
651 the counting grid or touched the inclusion lines (green), but not the exclusion ones (red).  
652 The cells counted in this example are pointed with arrowheads; bar = 9  $\mu\text{m}$ .

653 Figure 3 – Relative volume of liver collagen in male and female young, adult, middle-  
654 aged and old rats. Data expressed as mean  $\pm$  standard deviation; statistically significant  
655 differences between genders (\*) and to young animals ( $\Psi$ ).

656 Figure 4 – Total number (N) and number per gram (N/g) of hepatic stellate cells in male  
657 and female young, adult, middle-aged and old rats. Data expressed as mean  $\pm$  standard  
658 deviation; (\*) statistically significant differences between genders.

659 Figure 5 – Number-weighted mean volume of hepatic stellate cells in male and female  
660 young, adult, middle-aged and old rats. Full lines refer to the volume of whole cell and  
661 dotted lines to the cell body. Data expressed as mean  $\pm$  standard deviation; ( $\Psi$ ,  $\gamma$ )  
662 statistically significant differences to old animals.

663 Figure 6 – Total number (N) and number per gram (N/g) of hepatocytes (mononucleate  
664 and binucleated) and of binucleated hepatocytes in male and female young, adult,  
665 middle-aged and old rats. Data expressed as mean  $\pm$  standard deviation; statistically  
666 significant differences between genders (\*) and age groups ( $\gamma$ ).

667 Figure 7 – Number-weighted mean volume of mononucleate and binucleated  
668 hepatocytes in male and female young, adult, middle-aged and old rats. Data expressed  
669 as mean  $\pm$  standard deviation; (\*) statistically significant differences between genders.

670 Figure 8 – Total number (N) and number per gram (N/g) of hepatocytes in male and  
671 female young, adult, middle-aged and old rats. Data expressed as mean  $\pm$  standard  
672 deviation; (\*) statistically significant differences between genders.

673 Figure 9 - Total number (N) and number per gram (N/g) of liver sinusoidal endothelial  
674 cells in male and female young, adult, middle-aged and old rats. Data expressed as  
675 mean  $\pm$  standard deviation; (\*) statistically significant differences between genders.

676 Figure 10 – Estimated cell ratios of hepatocytes, Kupffer cells, hepatic stellate cells and  
677 liver sinusoidal endothelial cells in male and female rats (considering all age groups).

678

679 **Supplemental figures**

680 Supplemental Figure 1 - Thick section of an adult male liver immunostained against  
681 glial fibrillary acidic protein. Hepatic stellate cells (arrows) can be seen around the  
682 portal tract (P) and central veins (V). For unequivocal identification of these, glutamine  
683 synthetase immunomarking (that tags pericentral hepatocytes) was also used.

684 Supplemental Figure 2 - Thin section of old male (A) and old female (B) rat liver  
685 immunostained against  $\alpha$ -smooth muscle actin. Immunomarking is restricted to the wall  
686 of blood vessels in portal tracts (A) and in the central vein (B), without noticeable  
687 positive cells alongside the sinusoids; bar = 30  $\mu$ m.