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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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Running title: Male and female differences in the liver throughout ageing.

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Abstract

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

Introduction

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

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

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

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

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

Materials and Methods

Animals

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

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

Tissue Preparation

Sampling was performed during the morning period (from 10:00 to 12:00), to circumvent oscillations in liver function due to circadian rhythmicity (Davidson et al. 2004). In females, daily vaginal cytologies were observed, in order to avoid collecting samples in proestrous/oestrous days. Beforehand, animals were deeply anaesthetised with ketamine plus xylazine. Blood was collected and centrifuged to obtain serum for assessing alanine transaminase and aspartate transaminase levels. Transcardiac perfusion was performed with an isosmotic solution; the liver was weighed and its volume determined by the Scherle's method, as detailed elsewhere (Marcos et al. 2012). A smooth fractionator sampling scheme was applied (Marcos et al. 2012): half of the paraffin blocks was used for thick sections (30 µm thick) and exhaustively sectioned, whilst the other half was used for thin sections (3 µm thick). Of the thick sections, five sections in every 30 were sampled in order to immunostain against: 1) glial fibrillary acidic protein for estimating the N and N/g of HSC; 2) ED2 for estimating the N and N/g of KC; 3) E-cadherin, to differentiate mononucleate HEP from binucleated HEP, estimating their percentage, and to assess the N and N/g of HEP; 4) Von Willebrand Factor to estimate the N and N/g of LSEC; 5) glial fibrillary acidic protein and glutamine synthetase [an established marker of centrilobular HEP (Gebhardt and Mecke 1983)] to evaluate the lobular distribution of HSC. Thin sections were used for immunostaining against: 1) α-smooth muscle actin to evaluate the existence of activated HSC; 2) glial fibrillary acidic protein to determine the relative volume of HSC (whole cell); 3) glutamine synthetase, to estimate the lobular size, by measuring the porto-central distance. Thin sections were also used for assessing liver collagen, by Sirius red staining. In addition, tiny liver fragments (< 0.5 mm^3) were removed from the rat liver for electron microscopy. These were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH = 7.4) for two hours and subsequently post-fixed in phosphate buffered 1% OsO_4 for another two hours. After dehydration in ethanol and propylene oxide, the pieces were embedded in epoxy resin. Semithin sections were obtained and stained with methylene blue-azur II, which were used to quantify volume densities of HSC (cell body). Additionally, ultrathin sections were obtained, contrasted with uranyl acetate and lead citrate and observed in a transmission electron microscope, JEOL 100CXII, at 60 kV.

Immunohistochemistry

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

For the double immunohistochemistry, slides were also placed in the microwave (this time for three cycles of four minutes at 600W in buffered citrate). After blocking endogenous biotin and peroxidase, the first streptavidin–biotin protocol was followed with antibody against glial fibrillary acidic protein (1:1500 dilution for four days at 4ºC). Slides were developed for two minutes in 0.05% 3,3'-diaminobenzidine (Dako) in Tris-buffered saline with 0.03% H_2O_2. Sections were then rinsed in tap-water and dipped in 50 mM glycine buffer (pH = 2.2) for five minutes, to strip off the antibodies of the first immunoreaction. The second streptavidin–biotin protocol against glutamine synthetase followed, using 1:4000 rabbit polyclonal antibody (graciously provided by Professor Rolf Gebhardt, University of Leipzig), for another four days at 4ºC. Slides were developed with aminoethylcarbazole (Dako) for 10 to 20 minutes (final red colour controlled by microscopic observation) and mounted in Aquatex (Dako).

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

immunostaining, slides were placed in a pressure cooker for three minutes in citrate buffer (pH = 6.0). After rinsing in phosphate buffered saline, a polymer based immunohistochemical protocol was followed [Novocastra Novolink Polymer (Leica Biosystems, Newcastle, United Kingdom)]. The protein blocking solution was applied for five minutes and α-smooth muscle actin antibody (clone HM45, Dako) diluted at 1:500 immersed the slides overnight. The post-primary solution and polymer were both applied for 30 minutes. Finally, slides were developed for two minutes in diaminobenzidine. For assessing the porto-central distance, immunohistochemistry against glutamine synthetase was used (diluted at 1:4000), following the protocol for glial fibrillary acidic protein immunostaining in thin sections.

Positive and negative controls (omission of first antibody and replacement by non-immune serum) were included, both in thin and thick sections, and all slides were evaluated blindly (*i.e.*, the observer was unaware of the gender or age of the animal), to avoid eventual observer-related bias.

Histochemistry with Sirius-red

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

Morphometrical Analysis

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

Stereological Analysis

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

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

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

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

Statistical analysis

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

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

Results

Qualitative findings

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

Quantitative findings

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

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

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

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

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 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 of HEP was statistically correlated with *i*) body and *ii*) liver weights ($r = 0.60$; $p < 0.0001$, for both) and *iii*) with the N of KC ($r = 0.5$, $p < 0.01$), whereas the N/g of HEP was correlated with the N/g of KC and N/g of binucleated HEP ($r = 0.94$, $p < 0.001$ and $r = 0.75$, $p = 0.02$, respectively). Gender differences were observed in the N/g of HEP, but these were restricted to younger animals ($136 \pm 11 \times 10^6$ in males and $183 \pm 39 \times 10^6$ in females) ($p = 0.016$). Similarly, gender differences existed for binucleated HEP, as well as their percentage, which were higher in females ($25 \pm 4\%$ in young males and $34 \pm 5\%$ in young females), but these differences were attenuated with ageing ($27 \pm 5\%$

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

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

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

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

Discussion

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

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

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

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

However, it should be acknowledged that two-dimensional counts ("numbers per area") are biased and may not reveal the three-dimensional reality of biological tissues (Mandarim-Lacerda 2003; Marcos et al. 2012). The larger cell body of an aged HSC could be prone to an overestimation bias in two-dimensional counts. The shift from a centrilobular to a periportal predominance of the HSC with ageing is another new finding. It has been reported that quiescent HSC are able to slowly move through the space of Disse (Senoo et al. 2007; Friedman 2008), being dynamic in the changeable 3D structure of the sinusoids (Sato et al. 2003; Senoo et al. 2007). It has long been known that the matrix composition differs along the porto-central axis (Reid et al. 1992; Roskams et al. 2007; Senoo et al. 2010). It is conceivable that an increase of factors that drive HSC migration, *e.g.* transforming growth factor-β1 (Yang et al. 2003), could occur with ageing. It is noteworthy that aged HSC were not activated, being α-smooth muscle actin antibody negative, as previously described (Warren et al. 2011). However, their shape was different with ageing. Classical and more recent studies have reported that HSC in aged animals appear swollen, having significantly more and larger lipid droplets than in young animals (Enzan et al. 1991; Warren et al. 2011). This was confirmed by us, since a significant increase in the number-weighted mean volume of the cell body occurred with ageing. Based on such differences, some authors concluded that aged HSC are larger than young HSC (Vollmar et al. 2002; Schmucker 2005); however our study suggests that, based on the number-weighted mean volume of HSC, the cells actually change from a small cell body with long and thin extensions in youngsters to a large cell body with thicker and much shorter extensions in older rats. Actually, this large cell body justifies the recommendation of using old animals for isolating HSC (Ramadori and Saille 2002; Tacke and Weiskirchen 2005; Friedman 2008) — since cells contain more lipid droplets they can be better separated by gradient centrifugation. Moreover, the larger cell body of HSC should also contribute to the blood flow reduction in sinusoids of older animals (Vollmar et al. 2002; Warren et al. 2011) because it protrudes into sinusoids — as has previously been reported (Warren et al. 2011) — and HSC shift to periportal areas. It may be hypothesized that the shorter processes of aged HSC should surround and control the blood flow of fewer sinusoids than in youngsters, in whom HSC encircle more than two sinusoids (Friedman 2008).

Whether or not hyperplasia and/or hypertrophy of HEP occur with ageing remain 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

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

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

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

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

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

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

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

Figure 5 – Number-weighted mean volume of hepatic stellate cells in male and female young, adult, middle-aged and old rats. Full lines refer to the volume of whole cell and dotted lines to the cell body. Data expressed as mean ± standard deviation; (Ψ, γ) statistically significant differences to old animals.

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

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

Figure 8 – Total number (N) and number per gram (N/g) of hepatocytes in male and female young, adult, middle-aged and old rats. Data expressed as mean ± standard 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 ± 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

Supplemental figures

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

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