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Expression pattern of CYP24 in liver during ageing in long-term diabetes

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ABSTRACT

Association of liver calcitriol (active vitamin D metabolite) catabolism with osteomalacia during prolonged use of certain drugs was reported in several recent studies. To examine whether the increased calcitriol catabolism could be a potential link between ageing/diabetes mellitus (DM) and bone loss, we studied the dynamic of expression of CYP24, the main calcitriol catabolising enzyme in the liver of rats during ageing and a long-term experimental DM1. DM1 model was induced with intraperitoneally injected streptozotocin (STZ) (55 mg/kg). Sprague-Dawley rats were sacrificed 6 and 12 months after the DM1 induction. The immunohistochemical analyses of CYP24 and transforming growth factor β 1 (TGF-β1) expression in the liver were performed. We found that ageing and long-term DM1 resulted in a significantly increased expression of CYP24 in hepatocytes, as well as in non-hepatocyte liver cells (Kupffer cells, hepatic stellate cells and sinusoidal endothelial cells). Ageing and long-term DM1 resulted in an increased expression of TGF-β1 as well. Expression of CYP24 coexisted with the expression of TGF-β1 in all types of hepatic cells. We concluded that liver has the capacity for an active vitamin D catabolism in different populations of liver cells, especially in sinusoidal endothelial cells, through an expression of CYP24. That capacity is substantially increased during ageing and long-term diabetes mellitus. Increased liver calcitriol catabolism could be one of the mechanisms of the bone metabolism impairment related to ageing and diabetes.

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1. Introduction

Among many well described functions, the skeleton serves as the store/source of minerals for balancing blood and tissue ion content (Rosen et al., 2012). It is well known that ageing dramatically affects both the quality and quantity of mineralized bone, making bone-related diseases one of the most important health problems in elderly population of industrialized countries (Cameron and Demerath 2002; Saller et al., 2008). This population is commonly affected by chronic metabolic diseases (Morley, 2008). Among them, diabetes mellitus (DM) has increasing incidence worldwide (Wild et al., 2004). Both DM type 1 and 2 have been related to the impairment of bone quality and with the increased risk of fractures (Janghorbani et al., 2007). Adverse effects of DM on the skeleton of the ageing mice have been proved experimentally (Portal-Núñez et al., 2015).

Bone and mineral metabolism is regulated by complex interplay of different regulation systems, among which vitamin D plays a prominent role (Holick, 1996). An active vitamin D metabolite $1,25(OH)_2D3$, calcitriol, exerts important biological effects through an activation of the vitamin D receptor (VDR) (Jones et al., 1998; Carlberg and Polly, 1998).

Metabolic activation of vitamin D begins in the liver by synthesis of 25-hydroxyvitamin D3 (25(OH)D3), the major circulating form of vitamin D (Haussler et al., 2013). The 25(OH)D3 is a substrate for the mitochondrial enzyme 25-hydroxyvitamin D3-1α-hydroxylase (1α-hydroxylase, 1α-OHase, CYP27B1), that catalyzes conversion of 25(OH)D3 to calcitriol ($1,25(OH)_2D3$), the most active vitamin D metabolite (Dusso and Brown, 1998; Haussler et al., 1998). This reaction occurs primarily in the kidney, but it has also been detected in different extra-renal tissues (Townsend et al., 2005). Enzyme CYP24 catalyzes the initial step in the conversion of calcitriol to

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less active metabolites (Inouye and Sakaki, 2001; Swami et al., 2011). CYP24 is a multifunctional 24-hydroxylase (24-OHase) and the major catabolic enzyme of vitamin D (Brown et al., 1999). It is directly upregulated by VDR, and it is expressed mainly in the kidney (Armbrecht et al., 2003). CYP24 enzyme is expressed at a very low level in human liver and in small intestine (Gascon-Barre et al., 2003; Xu et al., 2006).

Studies have shown accumulating evidence of close interaction of vitamin D and transforming growth factor (TGF-β) signalling paths in animal models (Schnaper et al., 2003; Abramovitch et al., 2011; Ding et al., 2013). TGF-β is considered to be the key profibrogenic cytokine in the liver (Gressner et al., 2002). It mediates hepatic stellate cells (HSCs) activation, hepatocyte apoptosis, and extracellular matrix formation and it also induces expression of other profibrogenic mediators (Weng et al., 2009). TGF-β expression is increased in liver damaged by DM in animal models (Sahai et al., 2004; Halici et al., 2009). As a central mediator of fibrogenic remodelling processes in the liver, TGF-β1 isoform expression is an indicator of liver cells damaged by the inflammation (Weng et al., 2007).

Findings of increased calcitriol degradation in liver and intestines were proposed to play an important role in pathophysiological mechanism for development of osteomalacia related to the long-term therapy with some antiepileptic drugs (Pascussi et al., 2005; Zhou et al., 2006). However, experimental evidence regarding the role of different calcitriol-catabolising enzymes is still inconsistent (Pascussi et al., 2005; Zhou et al., 2006). Different mechanisms of age- and DM-related bone impairment have been described (Wongdee and Charoenphandhu, 2011; Yamaguchi and Sugimoto, 2012). However, the possible role of elevated calcitriol degradation by increased catabolic liver enzymes in pathophysiology of these conditions was not studied. Hence, we investigated the dynamic of CYP24 expression in rat liver during ageing and long-term DM1. To examine whether the CYP24 expression (and the extent of calcitriol catabolism) is related to development of inflammatory processes in the liver, we also studied the co-expression of CYP24 with a leading inflammatory mediator, TGF-β1.

2. Materials and methods

2.1. Ethics

All experimental procedures and protocols were approved by the Ethical Committee of the University of Split School of Medicine.

2.2. Animals

Male Sprague-Dawley rats weighing between 160 and 200 g were used. All rats were raised under controlled environmental conditions (temperature 22 ± 1 °C; lighting schedule: 12 h of light and 12 h of dark) at the University of Split animal facility. Animals were reared individually in plastic cages with sawdust bedding. Five groups of rats were included: 3-month-old control (c-3m; N = 4), 8-month-old control (c-8 m; N = 7) and diabetic group (dm-8 m; N = 7); and 14-month-old control (c-14 m; N = 7) and diabetic group (dm-14 m; N = 6). Duration of diabetes was 6 or 12 months for dm-8 m or dm-14 m group, respectively.

2.3. Diabetes induction

In order to induce DM type 1 (DM1) model, animals were injected intraperitoneally (i.p.) with 55 mg/kg of streptozotocin (STZ) freshly dissolved in citrate buffer (pH 4.5) after overnight fasting (Ferhatović et al., 2013). Age-matched control group for the DM1 model was injected i.p. with pure citrate buffer solution. Animals were divided into two groups, based on duration of diabetes from injection of STZ until the end of experiment (6 months and 12 months). Each diabetic group was matched with a control group of the same age. Both groups were fed *ad libitum* with normal laboratory chow (4RF21 GLP, Mucedola srl, Settimo Milanese, Italy).

Plasma glucose levels were measured with a glucometer (One-Touch Vita, LifeScan, High Wycombe, UK). The initial glucose measurement was performed on post-injection day 3 or 4. Glucose level over 300 mg/dl was considered a confirmation of successfully induced DM1. Diabetic rats received 1 U of long-acting insulin (Lantus Solostar; Sanofi-Aventis Deutschland GmbH, Frankfurt, Germany) weekly to prevent ketoacidosis.

2.4. Tissue collection and immunohistochemistry

Rats were anesthetized with isoflurane (Forane, Abbott Laboratories, Queenborough, UK) and sacrificed by decapitation. The liver tissue was removed and post-fixed in Zamboni's fixative (Zamboni and de Martino, 1967), dehydrated and embedded in paraffin wax. After deparaffinization, sections were rehydrated in ethanol and water, briefly rinsed with distilled water, heated in Dako Target Retrieval Solution (S2367 Dako Cytomatiom, Carpinteria, CA USA) for 12 min on 95 °C in microwave oven. After being cooled to the room temperature, sections were incubated overnight with primary antibody using standard immunohistochemical methods. Incubation with primary goat polyclonal antibody was used overnight for detection of CYP24 (1:100, sc-32165, Santa Cruz, CA, USA). Visualisation of primary antibody was performed using donkey anti-sheep FITC-conjugated antibody (1:200, 110217, Jackson ImmunoResearch Laboratories, Inc., Baltimore, PA, USA) or donkey anti-goat rhodamine-red conjugated antibody (1:300, 107828, Jackson ImmunoResearch Laboratories, Inc., Baltimore, PA, USA). Primary rabbit polyclonal antibody was used for detection of TGF-β1 (1:10, ab92486, Abcam, Cambridge, UK). Visualisation of primary antibody was performed using donkey anti-rabbit FITC-conjugated antibody (1:200, 110217, Jackson ImmunoResearch Laboratories, Inc., Baltimore, PA, USA).

After rinsing in PBS, nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). All slides were mounted, air-dried, and cover slipped (Immu-Mount, Shandon, Pittsburgh, PA, USA). Staining controls included omission of primary antibody from the staining procedure, and this resulted in no staining in liver tissue.

After deparaffinization and rehydration, endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 for 15 min, followed by heating in Dako Target Retrieval Solution (S2367 Dako-Cytomation, Carpinteria, CA, USA) for 12 min on 95 °C in microwave oven. After cooling to the room temperature, slides were washed with PBS and then incubated overnight in primary CYP24 antibody. After washing in PBS, visualisation of primary antibodies was performed using LSAB+ System-HRP K0690 (DakoCytomation, Carpinteria, CA, USA), by manufacturer's instructions. Detection was performed by using diaminobenzidine (DAB) reaction (Liquid DAB+ Substrate chromogen system K3468; DakoCytomation, Carpinteria, CA, USA). Nuclei were counterstained with hematoxyline. Sections were then prepared for the light microscope viewing (BX51, Olympus, Tokyo, Japan).

Alternatively, after deparaffinization, sections were stained by hematoxylin and eosin.

2.4.1. Double immunolabelling

To determine the identity of cell populations that express CYP24, double immunofluorescence staining was used. Combination of anti-CYP24 (1:100, sc-32165, Santa Cruz, CA, USA) and anti-glial fibrillary acidic protein (GFAP)-antibody (1:300, ab7260,

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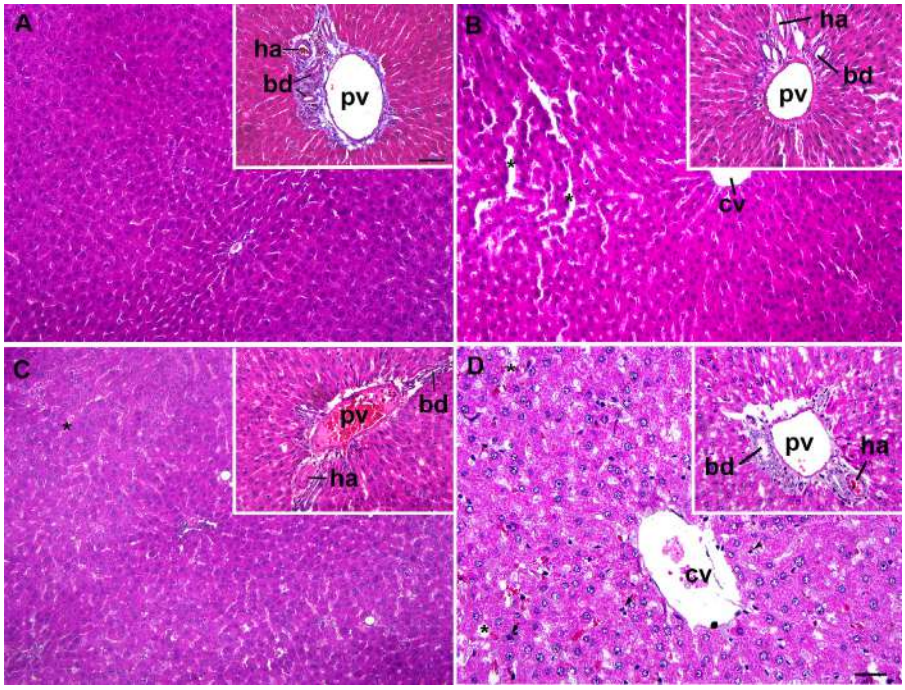

Fig. 1. Representative hematoxylin-eosin micrographs of liver tissue sections from c-8 m (A), dm-8 m (B), c-14 m (C), and dm-14 m (D). Control (c-8 m) had normal histological structure of the central veins (cv) and surrounding hepatocytes in the hepatic parenchyma (A). Diabetic groups at the age of 8 and 14 months displayed mild congestion of liver, dilatation of the central veins, sinusoidal spaces (*), the portal vein branches (pv) and the bile ducts (bd) in the portal triad (insets), branches of hepatic artery (ha), Kupffer cells (arrows), hepatic stellate cells (arrowhead). Mild degeneration and fatty changes of hepatocytes can be observed in both c-14 m and dm-8 and dm-14 (B–D). Scale bar for insets = 40 μm; A–D = 20 μm.

rabbit, Abcam, Cambridge, UK), was used, followed by donkey anti-goat rhodamine-red conjugated antibody and donkey anti-rabbit FITC-conjugated antibody (1:300, 107828 and 1:200, 110217 respectively; both Jackson ImmunoResearch Laboratories, Inc., Baltimore, PA, USA). Entire liver section areas of all experimental animals were examined for existence of co-localization in specific cell populations.

2.5. Data acquisition and analysis

Liver sections were viewed and photographed using a microscope (BX51, Olympus, Tokyo, Japan) equipped with cooled digital camera (DP71, Olympus, Tokyo, Japan) and processed with Cell A, Imaging Software for Life Sciences Microscopy (Olympus, Tokyo, Japan). CYP24 immunoreactive cells were counted as number of cells in entire section area. Each microphotograph of liver section taken at ×40 objective magnification was divided into squares of 100 × 100 μm. By using Image J software, each photograph was divided into squares of 100 × 100 μm. The squares were randomly assigned to microphotographs, by using automatic Image J random-assignment option through which a network of squares was set randomly on each additional microphotograph. Every second square on each image was analyzed. Six selected fields were analyzed in each per six microphotographs. CYP24 immunoreactive cells were counted as number of cells in entire section area. The hepatocytes with immunoreactive nuclei were considered as 'CYP24 positive nuclei' and the hepatocytes with immunoreactive cytoplasm were considered as 'CYP24 positive cytoplasm'. The measured area was expressed per mm^2. The total number of immunoreactive non hepatocytes cell (nhc) was counted for each image and the percentage of CYP24 positive non hepatocytes cells in control and diabetic group was expressed as mean (M) ± standard deviation (SD).

For quantification of TGF-β1 expression, four fields were captured, using 40× objective. Each field was a single image. Measurement of threshold area was performed by using Image J on microphotographs. The threshold area % was calculated from the threshold area and the total surface of the microphotograph.

For quantification of CYP24-TGF-β1 co-localized cells expression, two fields were captured using 100× objective. Separate images were acquired under the same conditions for all fields using filters that allow visualisation of TGF- β1—fluorescein isothiocyanate (FITC), CYP24—Rhodamin (R) and nuclei—4′,6-diamidino-2-phenylindole (DAPI). Two selected fields were analyzed in each per two merged microphotographs. CYP24-TGF-β1 co-localized cells were counted as number of cells in entire section area. The total number of immunoreactive CYP24-TGF-β1 co-localized cells was counted for each image and the percentage of CYP24-TGF-β1 co-localized positive cells in control and diabetic group was expressed as M ± SD.

2.6. Statistical analysis

For statistical analyses one-way ANOVA followed by *post hoc* Tukey test, and factorial ANOVA were performed using statistical software Statistica 7.0 (StatSoft, Tulsa, OK, USA). Data were presented as M ± SD. Statistical significance level was set at $p < 0.05$.

3. Results

3.1. General remarks

Hematoxylin and eosin staining of the liver sections obtained from control groups (c-8m) showed the typical normal hepatic histology and normal hepatic parenchyma (Fig. 1A). There was a mild morphological alteration detected in the older control group (c-14m) (Fig. 1C). Degeneration and fatty infiltration of hepatocytes were observed in c-14 m as well as in the diabetic groups (dm-8 m and dm-14 m). Additionally, diabetic groups at the age of 8 and 14 months displayed dilatation of the central veins, the portal vein

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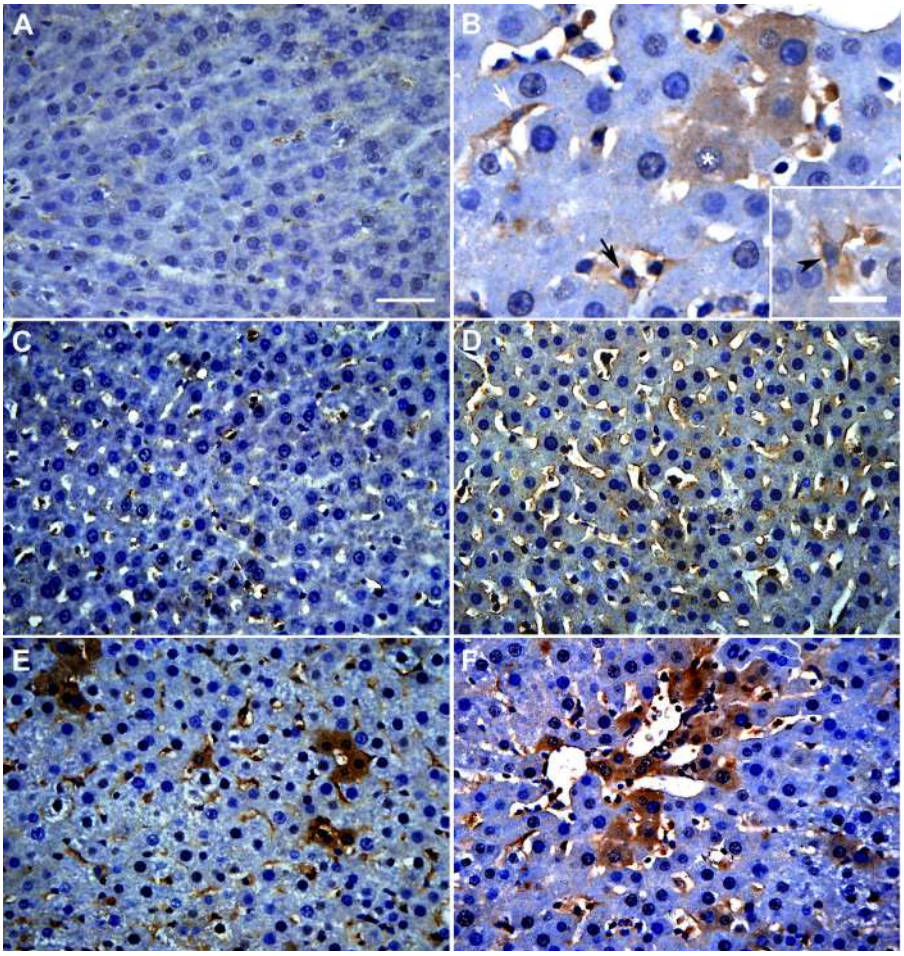

Fig. 2. Changes in expression of CYP24 in the liver during ageing and long-term diabetes. Histological section through the liver of the control animals at the age of 3 months. DAB immunohistochemistry revealed very weak staining of CYP24 in cytoplasm, as well as in the nuclei of hepatocytes and sinusoidal endothelial cells (A). Histological section through the liver of the control animals at the age of 8 months. DAB immunohistochemistry revealed very weak staining of CYP24 in cytoplasm and nuclei of hepatocytes and sinusoidal endothelial cells (C), while histological section through the liver of the diabetic group (dm-8 m) revealed moderate to strong CYP24 staining in cytoplasm and the nuclei of the same cells (D). Liver of control (E) and diabetic animals at the age of 14 months (F) shows very strong expression of CYP24 staining in the cytoplasm and the nuclei of liver cells in the dm-14 m in comparison to the control. Details from dm-14 m group were shown on the panel (B). Histological section through the liver of the diabetic group (dm-14 m) revealed strong expression of CYP24 in the cytoplasm of liver sinusoidal endothelial cells (white arrow), hepatic stellate cells (black arrow), and Kupffer cells (arrowhead in the inset), while strong expression of CYP24 can be seen in both cytoplasms and nuclei of the hepatocytes (asterisk). Scale bar A, C–F = 40 μm; B = 20 μm.

branches and the bile ducts in the portal triads (Fig. 1B and D). Occasional mononuclear leukocytes cells as well as Kupffer cells (KCs) were detected in the hepatic parenchyma between the degenerated and fatty changed hepatocytes (Fig. 1D).

3.2. Expression of CYP24 in the rat liver during ageing and long-term diabetes

Immunohistochemical staining of CYP24 showed positivity in nuclei and cytoplasm of hepatocytes, liver sinusoidal endothelial cells (LSECs) and in specific population of small stellate cells localized in the space of Disse (HSCs) of both control and diabetic group of animals during aging. Expression of CYP24 in the control groups (c-3 m and c-8 m) displayed very weak staining in cytoplasm, as well as in the nuclei of hepatocytes and non hepatocyte cells (LSECs, HSCs and KCs), while in the liver of the diabetic group (dm-8 m) moderate to strong expression of CYP24 was observed in the cytoplasm of hepatocytes, LSECs, HSCs, and KCs (Fig. 2A–D). Diabetic animal group, at the age of 14 months, showed stronger expression of CYP24 staining in the cytoplasm and the nuclei of liver cells in comparison to the control group (Fig. 2B–F).

The number of CYP24-positive non hepatocyte cells was significantly higher in both diabetic group (dm-8 m and dm-14 m) in comparison to the control groups (c-8 m and c-14 m) ($p < 0.05$ and $p < 0.001$ respectively). During ageing, in the control groups of animals (c-3 m, c-8 m and c-14 m), the number of CYP24-positive non hepatocyte cells was significantly higher in the c-14 m ($p < 0.001$). Additionally, in the diabetic groups of animals during ageing, the number of CYP24-positive non hepatocyte cells was significantly higher in the dm-14 m compared to dm-8 m ($p < 0.001$) (Fig. 3A).

The percentage of CYP24-positive hepatocyte cytoplasms was significantly higher in both diabetic groups (dm-8 m and dm-14 m) in comparison to the control groups (c-8 m and c-14 m) ($p < 0.05$). During ageing, the number of CYP24-positive hepatocyte cytoplasms was significantly higher in the c-14 m group in comparison to the younger control groups of animals (c-3 m, c-8 m) ($p < 0.01$ and $p < 0.001$ respectively). In the diabetic groups of animals during aging the number of CYP24-positive hepatocyte cytoplasms was significantly higher in the dm-14 m compared to dm-8 m ($p < 0.01$) (Fig. 3B).

The ratio of CYP24-positive hepatocyte nuclei was significantly higher in dm-8 m compared to the c-8 m group ($p < 0.05$). During ageing, the number of CYP24 positive hepatocyte nuclei was sig-

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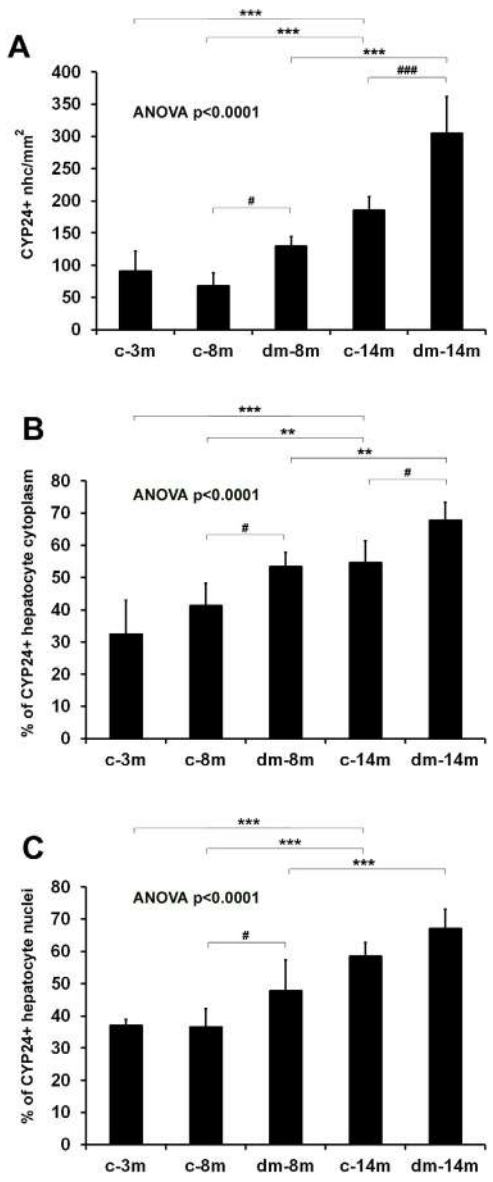

Fig. 3. Percentage of CYP24-positive non-hepatocyte cells (nhc) per square millimeters (A), percentage of CYP24 positive hepatocyte cytoplasm (B) and percentage of CYP24 positive hepatocyte nuclei (C) in different experimental groups. Data are presented as mean and SD, significant difference according to age ** $p < 0.01$, *** $p < 0.001$, significant difference between the diabetic and control groups # $p < 0.05$ and ### $p < 0.001$. 3-month-old control (c-3 m; N = 4), 8-month-old control (c-8 m; N = 7) and diabetic group (dm-8 m; N = 7); and 14-month-old control (c-14 m; N = 7) and diabetic group (dm-14 m; N = 6).

nificantly higher in the c-14 m group in comparison to the control groups of animals (c-3 m, c-8 m) ($p < 0.001$). In the dm-8m, the number of CYP24 positive hepatocyte nuclei was significantly higher than in the c-8 m ($p < 0.05$) (Fig. 3C).

3.3. Expression of TGF-β1 in the rat liver during ageing and long-term diabetes

In the control groups, expression of TGF-β1 in c-3 m displayed very weak staining while in c-8 m displayed moderate TGF-β1 staining (Fig. 4A and C). Threshold area percent (%) of TGF-β1 in the different experimental groups is shown in Fig. 4B. The liver of the diabetic group (dm-8 m) occasionally showed strong expression of TGF-β1, especially in the hepatocytes and sinusoidal endothelial cells (Fig. 4D). Expression of TGF-β1 was significantly higher in liver of 14-month-old control, in comparison to 3-month-old rats ($p < 0.001$; Fig. 4B). Diabetic animal group, at the age of 14 months, showed stronger expression of TGF-β1 staining in the cytoplasms and the nuclei of liver cells in comparison to the 3-month-old control group; $p < 0.001$ (Fig. 4B–F). In addition, significant increase in TGF-β1 expression was found in liver of diabetic (dm-14 m) animals at the age of 14 months, compared to dm-8 m rats ($p < 0.001$) (Fig. 4B–F).

3.4. Co-localisation of CYP24/TGF-β1 and CYP24/GFAP in liver tissue

Double immunofluorescence staining with CYP24 and TGFβ1 revealed co-localisation of these two markers in the Kupffer cells, hepatocytes and sinusoidal endothelial cells in all experimental groups. Representative examples were shown in Fig. 5A–C. As GFAP was proven to be a marker of HSCs we wanted to identify possible co-localization of CYP24 and GFAP markers in the same cells (Buniatian et al., 1996). HSCs also known as Ito cells (fat storing cells) play vital roles in regulation of the differentiation and proliferation of other hepatic cell types during liver regeneration. (Gulubova et al., 1999; Tennakoon et al., 2013). We found co-localisation of GFAP and CYP24 in the hepatic stellate cells (Fig. 5D).

During ageing, the percentage of CYP24 positive non hepatocyte cells was significantly higher in the c-14 m group in comparison to the younger control groups of animals (c-3 m, c-8 m) ($p < 0.001$). In the dm-8 m, the number of CYP24 positive nhc was significantly higher than in the c-8 m ($p < 0.01$). The percentage of TGF-β1 positive nhc in comparison to the younger control group of animals (c-3 m) was significantly higher in the c-8 m and c-14 m group ($p < 0.01$ and $p < 0.001$, respectively). The percentage of TGF-β1 positive hepatocytes was significantly higher in the c-14 m group in comparison to the younger control groups of animals (c-3 m, c-8 m) ($p < 0.001$). In the diabetic groups of animals with aging the number of TGF-β1 positive hepatocytes was significantly higher in the dm-14 m compared to dm-8 m ($p < 0.01$). The percentage of CYP24- TGF-β1 co-localized cells was significantly higher in comparison to the younger control group of animals (c-3 m) in the c-8 m and c-14 m group ($p < 0.001$; Fig. 6).

The interaction between age and DM was revealed by comparison of diabetic groups of rats in two different stages/ages (6-month DM and 12-month DM; 8 and 14 months old), concerning all investigated parameters. Additional two-way (factorial) ANOVA revealed significant time × dm effect for: number of CYP24+ nhc ($p < 0.05$); number of TGF-β1 + cells ($p < 0.0001$); and % of CYP24+ nhc ($p < 0.01$).

4. Discussion

Ageing and long-term DM are related to accumulation of pathological liver processes, resulting in damaged morphology and exchanged function (Wongdee and Charoenphandhu, 2011; Farrell and Larter, 2006). The influence of ageing and DM on liver vitamin D catabolism, being consequently potential link between these changes and bone loss, was not examined until now. Recent studies relating liver calcitriol catabolism with osteomalacia during prolonged use of certain drugs (Pascussi et al., 2005; Zhou et al., 2006) raised the question whether similar interaction could be a part of the pathophysiological mechanisms in development of senile and DM-related bone loss.

Hence, we studied the dynamic of expression of CYP24, the main calcitriol catabolising enzyme in the liver of rats during ageing and experimental DM1.

The 24-OHase (CYP24) enzyme plays an important role in attenuating vitamin D responsiveness by catalyzing the conversion of

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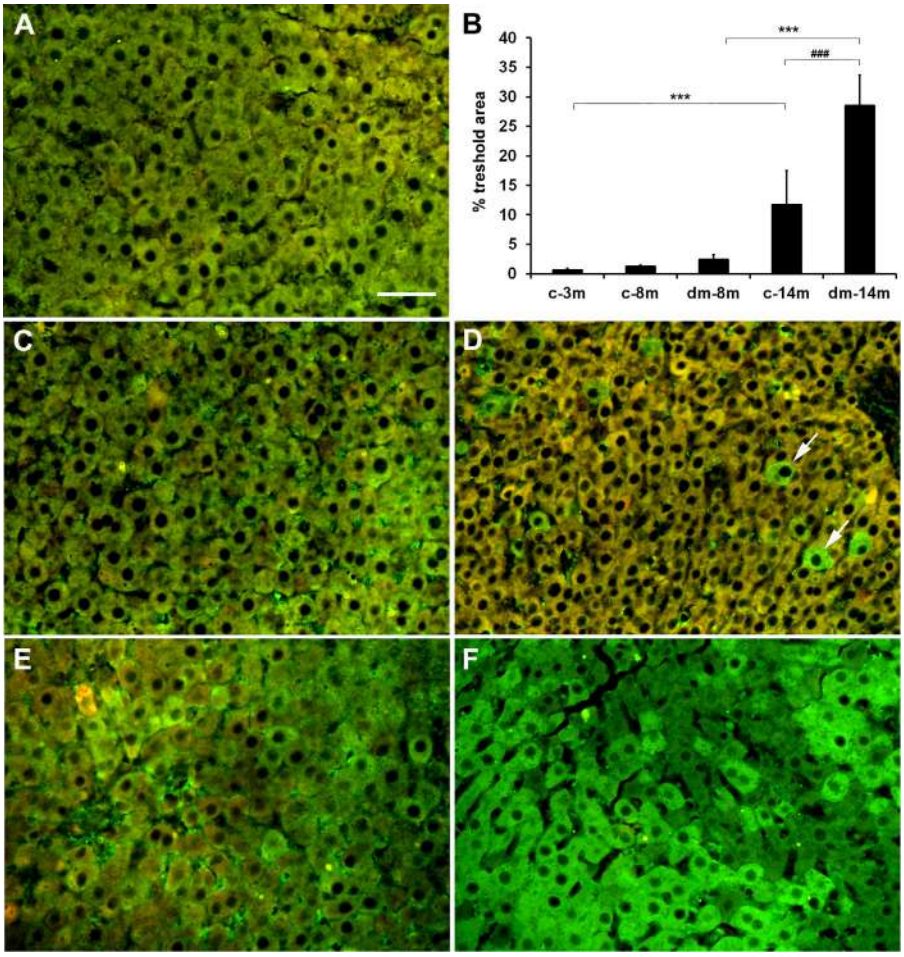

Fig. 4. Changes in expression of TGF-β1 in the liver during ageing and long-term diabetes. Histological section through the liver of the control animals at the age of 3 months. Immunofluorescence staining revealed very weak staining of TGF-β1 in the hepatocytes and sinusoidal endothelial cells (A). Threshold area percent (%) of TGF-β1 in different experimental groups. Data are presented as mean and SD, significant difference according to the age *** $p < 0.001$; significant difference between the diabetic and control groups ### $p < 0.001$ (B). Histological section through the liver of the control animals at the age of 8 months. Immunofluorescence staining revealed moderate staining of TGF-β1 in the liver cells (C), while histological section through the liver of the diabetic group (dm-8 m) revealed strong TGF-β1staining of the hepatocytes and sinusoidal endothelial cells especially in the certain hepatocytes (arrows) (D). Liver of control animals at the age of 14 months (E) show strong expression of TGF-β1 staining in the liver cells while diabetic (dm-14 m) (F) show very strong expression of TGF-β1 staining in the hepatocytes and sinusoidal endothelial cells in comparison to the control. Scale bar A, C–F = 40 μm. (3-month-old control (c-3 m; N = 4), 8-month-old control (c-8 m; N = 7) and diabetic group (dm-8 m; N = 7); and 14-month-old control (c-14 m; N = 7) and diabetic group (dm-14 m; N = 6).

calcitriol to less active 24-hydroxylated metabolites. Consistent with its role as a negative feedback enzyme, expression of 24-OHase is induced by $1,25(OH)_2D3$ itself through binding of liganded VDR and its heterodimeric receptor, the retinoid X receptor (RXR) to VDR-responsive elements in the promoter region of CYP24 (Ohyama and Okuda, 1991; Ohyama and Yamasaki, 2004). CYP24 is perhaps the most sensitively regulated target gene of $1,25(OH)_2D3$, and it seems likely that this feedback control mechanism will operate at most sites of $1,25(OH)_2D3$ synthesis and action (Kerry et al., 1996). Autocrine synthesis of $1,25(OH)_2D3$ during inflammatory diseases such as diabetes mellitus (George et al., 2012), sarcoidosis (Adams et al., 1983) and Crohn's disease (Abreu et al., 2004) is commonly dysregulated and may lead to increased circulating levels of $1,25(OH)_2D3$.

Using immunohistochemistry, we found an extensive expression of CYP24 in different hepatic cell types: hepatocytes, LSECs, KCs and HSCs of both control and diabetic group of animals. In hepatocytes, we observed CYP24 expression in cytoplasms, as well as in nuclei. Similarly to our results, an expression of this enzyme in nuclei was also observed in neurons of dorsal root ganglia (Tague and Smith, 2011).

Presence of CYP24 in different types of liver cells indicates that these cells might express the enzyme for degradation of calcitriol used for their own needs. That could explain nuclear localization of the enzyme. Namely, upon its binding to VDR in cytoplasm, calcitriol is being translocated to the nucleus in order to regulate expression of the target genes (Haussler et al., 1998; Dusso et al., 2005). It is possible that CYP24 located in nucleus serves to immediately start calcitriol degradation process and limit its further action. In line with this, it was reported that all types of hepatic cells express active VDR (Gascon-Barre et al., 2003; Vuica et al., 2015). In addition, it was observed that selective hepatic cell populations are targets for the vitamin D endocrine/paracrine/intracrine system (Gascon-Barre et al., 2003).

In the present study, we observed substantial increase in hepatic CYP24 expression related to both ageing and long-term DM1 in a rat model. This increase was observed in hepatocytes as well as non hepatocyte liver cells, including LSECs, KCs and HSCs. Observing hepatocytes, an increase in CYP24 expression was found in cytoplasms as well as in nuclei. A major beneficial role of calcitriol in reducing chronic liver degenerative diseases such as diabetes has been proposed (Hayes 2010; Najmi Varzaneh et al., 2013; Danescu et al., 2009). Increased CYP24 expression that we

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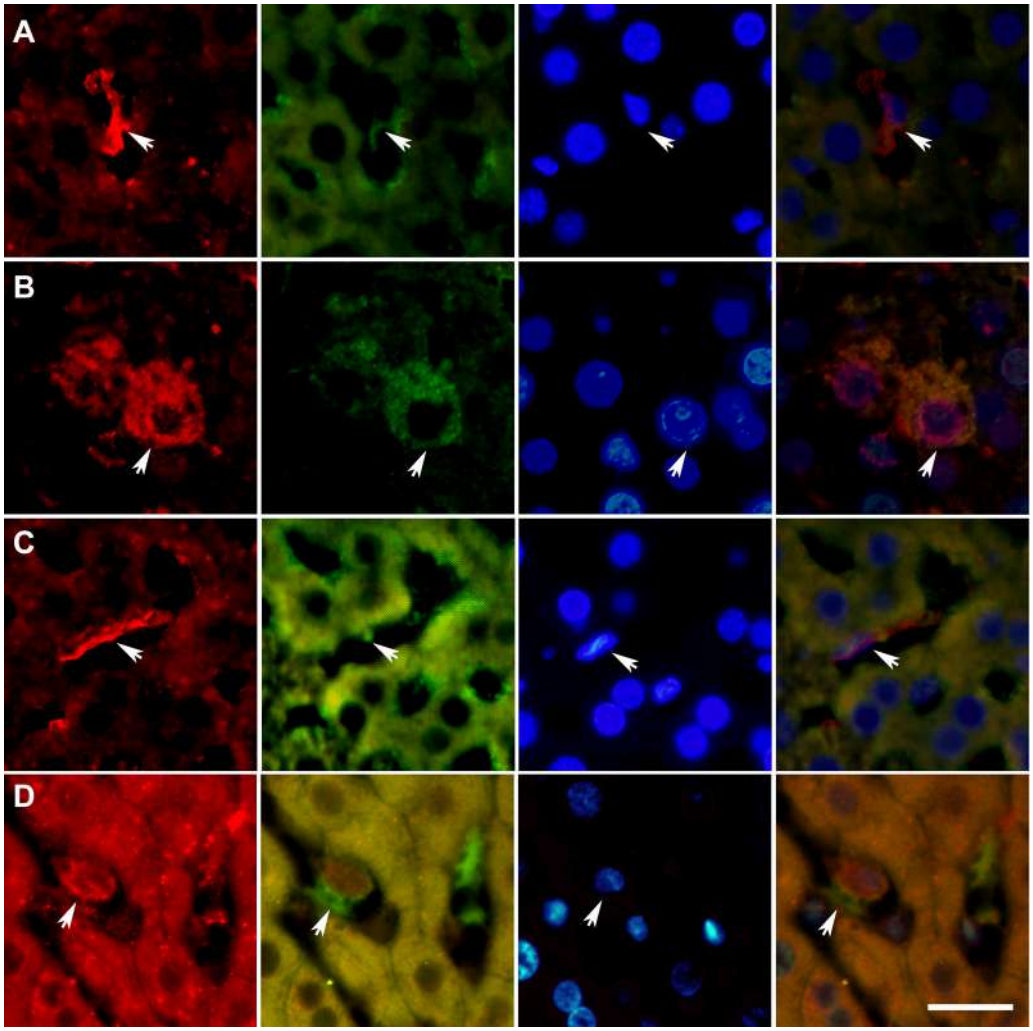

Fig. 5. Histological section through the liver of the diabetic group (dm-14 m). Entire liver section area of all experimental animals was examined for existence of co-localization in specific cell populations. Representative examples were shown. Co-localisation (arrows) of CYP24 (red) and TGF-β1 (green) in the Kupffer cell (A), hepatocyte (B), and sinusoidal endothelial cell (C). Co-localisation (arrow) of CYP24 (red) and GFAP (green) in the hepatic stellate cells (D). DAPI (blue staining of nuclei). Scale bar = 20 μm (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

observed during ageing and DM could influence the local calcitriol metabolism. Nevertheless, extensive calcitriol catabolism in hepatic cells, especially hepatocytes and LSECs could substantially influence systemic calcitriol concentrations and function, including renal mineral excretion, intestinal calcium absorption and bone remodeling (Haussler et al., 2013). LSECs are a cell type in the liver tissue mainly responsible for the clearance of the most colloids and soluble waste macromolecules from the circulation (Elvevold et al., 2004). Kawai et al. showed that the cells that most efficiently accumulated vital stain in the liver were the LSECs. These cells are extremely active in uptake of the soluble or colloidal materials (Kawai et al., 1998). In combination with KCs, LSECs constitute the most powerful scavenger system in the body (Elvevold et al., 2004). Hence, extensive CYP24 expression in LSECs could substantially influence systemic calcitriol metabolism.

The possible mechanism for observed ageing- and DM-related increase of CYP24 expression is not known. However, we recently reported a finding of the hepatic source of calcitriol in monocyte/macrophage hepatic system, which was, along with the VDR expression, substantially increased in liver of aged and diabetic rats (Vuica et al., 2015). The dynamic steady state of bioactive vitamin D form—calcitriol is dependent on its synthesis catalyzed by 1α-OHase, but it also depends on the extent of its degradation. Since the expression of CYP24 is directly upregulated by calcitriol/VDR, as a negative regulating loop (Armbrecht et al., 2003; Sutton and MacDonald, 2003) it is reasonable to suggest that an increased calcitriol production and VDR expression could cause the increased CYP24 expression. In line with this, recent studies also showed that KCs, HSCs and LSECs responded to calcitriol by a significant increase in the CYP24, indicating that the VDR is functional in these cells (Gascon-Barre et al., 2003).

To examine the extent of pathological changes during ageing and long-term diabetes we also investigated expression pattern of TGF-β1, as a key mediator of fibrogenic remodelling processes in the liver (Gressner et al., 2002; Weng et al., 2007). It is known that TGF-β1 mediates HSC activation, hepatocyte apoptosis, and extracellular matrix formation and induces expression of other profibrogenic mediators (Weng et al., 2009).

In the liver, TGF-β is a very potent profibrogenic mediator of cellular responses that lead to tissue repair, growth regulation, and apoptosis (Bissell et al., 2001). An aberrant expression of TGF-β is involved in a number of pathological processes including liver fibrosis and inflammation. This was demonstrated in transgenic mice, which develop multiple tissue lesions including hepatic fibrosis and hepatocyte apoptosis due to an over-expression of active TGF-β1 in the liver (Sanderson et al., 1995). The liver injury of any

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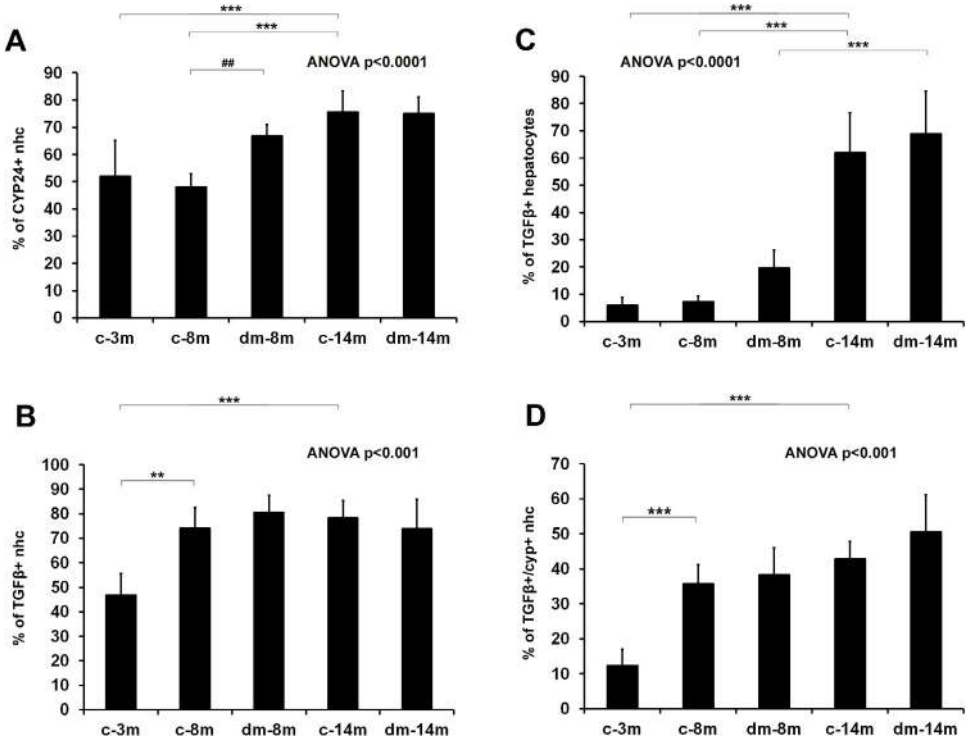

Fig. 6. Percentage of CYP24 positive non-hepatocyte cells (nhc) (A), percentage of TGF-β1 positive non hepatocyte cells (nhc) (B), percentage of TGF-β1 positive hepatocytes (C) and percentage of CYP24-TGF-β co-localized cells in different experimental groups. Data are presented as mean and SD, significant difference according to age ** $p < 0.01$, *** $p < 0.001$, significant difference between the diabetic and control groups ## $p < 0.01$; 3-month-old control (c-3 m; N = 4), 8-month-old control (c-8 m; N = 7) and diabetic group (dm-8 m; N = 7); and 14-month-old control (c-14 m; N = 7) and diabetic group (dm-14 m; N = 6).

etiology leads to activation of HSCs by transition of quiescent HSCs into proliferative, fibrogenic, and contractile myofibroblasts. Activated HSCs migrate and accumulate at the sites of tissue repair, secreting large amounts of extracellular matrix (ECM). Changes in the composition of the ECM can directly stimulate fibrogenesis. Type IV collagen and fibrinogen stimulate resident HSCs by activating latent cytokines, such as TGF-β1. Therefore, liver injury results in a rapid induction of TGF- β synthesis. Accordingly, diabetes-induced liver injury also includes inflammatory response and liver fibrosis. Hence, we used TGF-β immunoreactivity as reliable marker for detection of liver inflammatory processes. Several studies confirmed a role of the vitamin D-system in modulating the inflammatory response (Van Etten and Mathieu, 2005). Since we previously observed an increase in 1α-hydroxylase in the hepatic tissue of ageing and diabetic rats (Vuica et al., 2015) the aim of the present study was to investigate whether inflammatory milieu in these conditions is related to increased calcitriol catabolism as well, that could potentially have general influence on vitamin D metabolism.

We observed a substantial increase of TGF-β1 expression during ageing and long-term DM, confirming a progression of chronic inflammatory process. TGF-β1 was expressed in all types of hepatic cells, and its expression in cytoplasm of hepatocytes increased with ageing and progression of DM. An expression of TGF-β in hepatocytes itself is a matter of dispute (Schon and Weiskirchen, 2014). Hence, it is possible that hepatocytes during ageing and DM started to express TGF-β extensively, or this finding could result from increased accumulation of TGF-β produced by the other cell types in cytoplasm of hepatocytes. In our recent study (Vuica et al., 2015) we observed the occurrence of liver fibrosis in diabetes and age-damaged liver of rat, by using Trichrome staining. These findings are consistent with the appearance and expression of TGF- β in the studied groups.

We found an extensive co-expression of TGF-β1 with CYP24 in different types of hepatic cells. Especially, strong cytoplasmatic TGF-β1 expression in hepatocytes was co-localized with strong nuclear CYP24 expression. One could speculate that inflammatory changes in cells could result in increased CYP24 expression as well. Increased activity of CYP24 has been previously associated with other inflammatory diseases in humans. Namely, the expression of CYP24 was shown to be increased in renal tissue of patients with diabetic nephropathy (Helvig et al., 2010) and chronic kidney disease (Zehnder et al., 2008).

In conclusion, results of this study provide an evidence that the liver has a capacity for an active vitamin D catabolism in different populations of liver cells, especially in sinusoidal endothelial cells, through an expression of CYP24. We found that the expression of CYP24 is substantially increased during ageing and long-term diabetes mellitus in hepatocytes, as well as HSCs, KCs and LSECs. The results provide an evidence that increased liver CYP24 expression could be one of the mechanisms of the bone impairment related to ageing and diabetes, through its potential for extensive calcitriol catabolism.

Conflict of interest

The authors have no conflicts of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.acthis.2016.05.001.

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