Review

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Ageing, telomeres, senescence, and liver injury

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Populations in developed countries continue to grow older and an understanding of the ageing process to allow healthy ageing carries important medical implications. Older individuals are more susceptible to most acquired liver disorders and more vulnerable to the consequences of liver disease. Accordingly, age is a critical determinant of outcome for hepatitis C virus infection and liver transplantation. In this review we describe changes in the ageing liver and discuss mechanisms of senescence at the cellular level. In particular, we focus on mechanisms by which inflammation, oxidative stress, and oncogenic stress accelerate cellular senescence. In the setting of chronic hepatic injury and inflammation, cellular senescence functions as an essential stress-response mechanism to limit the proliferation of damaged cells and reduce the risk of malignancy, but this benefit is achieved at the expense of senescence-related organ dysfunction. The dual role of cell senescence in chronic liver disease will make this an intriguing but challenging area for future clinical interventions.

Introduction

According to Greek mythology, the Goddess Eos asked Zeus to grant immortality to the Trojan Tithonus, her mortal lover. But Eos had forgotten to ask for eternal youth so Tithonus received the "gift" of immortality and continued to age, withering beyond recognition and begging eternally for death (Fig. 1). In similar fashion, modern medicine, combined with improved sanitation and better nutrition, has increased the life expectancy of individuals in the developed world, but this has not been matched by progress in alleviating the ill health associated with increased age.

An increasing proportion of the healthcare budget is devoted to the growing geriatric population, so it is essential to understand the molecular basis of ageing and identify possible avenues for therapeutic intervention [1]. Clearly, patients of the same age can vary in terms of physiological reserve and susceptibility to disease in the absence of co-morbidity. The healthiest 70-year olds with the longest life expectancy within the ageing population have the lowest future health costs, so the solvency of modern medical heath care lies not just in extending patient survival but extending disease free survival.

Studies of healthy human ageing have relied largely on the study of healthy elderly individuals or by studying younger individuals afflicted with one of the Progeria syndromes associated with accelerated and premature ageing [2,3]. However, both approaches are affected by methodological problems. The former, by 'survivor bias' [4], i.e. those individuals who have survived to old age may show retarded features of biological ageing compared to those who have died; the latter, by a lack of understanding of the underlying pathophysiology and its relevance to normal human ageing.

The incidence of liver disease increases with age while the ability to withstand a hepatic insult falls with each decade. The cellular and sub-cellular changes that underlie this predisposition are the subject of much scrutiny. Interest in the role of ageing within the sphere of hepatology has increased, especially with the recent recognition of the critical importance of age in determining the clinical outcome in chronic hepatitis C virus infection [5] and the influence of donor age on graft survival after liver transplantation [6]. Furthermore, liver-related death in older persons is increased substantially when compared to younger individuals with the same condition [7].

There is irrefutable evidence that the ageing process occurs at the cellular level and that such changes can be induced 'prematurely' by inflammation. Cellular senescence is an important stress related response to injury that induces stable cell-cycle arrest with important implications for survival of the cell, organ, and organism by preventing replication of a cell in which the integrity of DNA has been compromised. The award of the 2009 Nobel prize for Medicine to Blackburn, Greider, and Szostak for research in telomere biology has brought the field of ageing research to the fore.

Hepatocyte changes with age

Hepatocyte structure changes with age. The volume of hepatocytes increases with development and maturation but declines with senescence [8,9]. The relative volumes of hepatocyte organelles also change during ageing.

The most common change on diagnostic liver biopsy specimens is age-related cytoplasmic accumulation of highly oxidised insoluble proteins, known as lipofuscin (Fig. 2A) [10,11]. Lipofuscin can be seen on H & E staining, are PAS diastase resistant and do not stain with Chromotrope Aniline Blue (Fig. 2B and C). These

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Fig. 1. Eos and Tithonus. The Goddess Eos abducting the Trojan Tithonus in his youth portrayed traditionally holding a lyre.

accumulations of highly cross-linked protein are thought to relate to chronic oxidative stress and a failure to degrade damaged and denatured proteins [11]. Increasing evidence suggests that lipofuscin interferes with cellular pathways due to its ability to trap metallic cations and facilitate further free radical formation [12].

Other sub-cellular hepatocyte changes with age are less well described and most data are derived from animal studies. There is a marked decline in smooth endoplasmic reticulum surface area with age [9,13] which correlates with decreased hepatic microsomal protein concentrations and enzymatic activity such as glucose-6-phosphatase [13]. Recent data suggest there is an age-related decline in chaperone-mediated autophagy, the cellular pathway leading to degradation of molecules and sub-cellular organelles. In a mouse model associated with age-related decline in liver function, restoration of autophagy was associated with maintenance of liver function [14].

There are also data suggesting a change in hepatocyte nuclear morphology with increasing age. It is known that there is increased variation in nuclear size [15], associated with an increasing incidence of polyploidy of hepatocytes [16]. After the age of 85, around 27% of human hepatocytes demonstrate polyploidy compared to around 6% for individuals in their twenties [17].

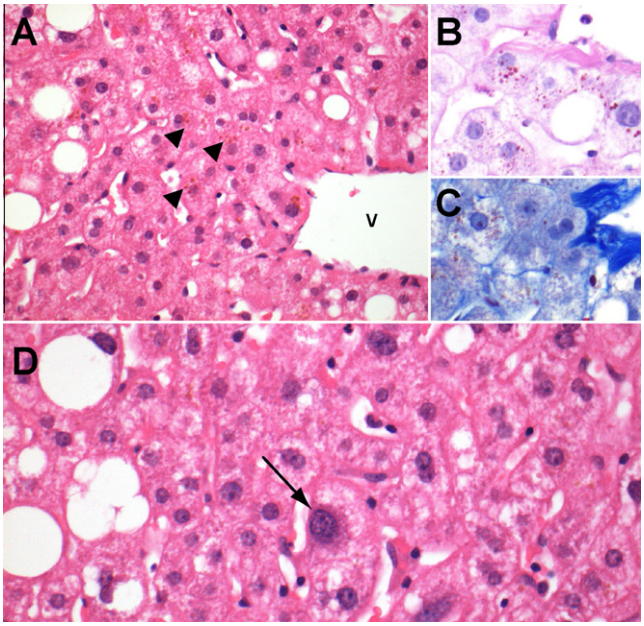

Fig. 2. Age-related changes seen on liver biopsy specimens include accumulation of lipofuscin (A–C) and large cell change (D). Liver biopsy specimen taken from a 72 year-old male with NAFLD stained with (A) haematoxylin and Eosin (H&E), diastase periodic-acid Schiff (dPAS) (B) and Chromotrope Aniline Blue (CAB) stain demonstrating intra-cytoplasmic granules of lipofuscin (arrow heads) adjacent to an hepatic venule (v) confirmed as dPAS resistant (B) but negative on the CAB stain (C). (D) Large cell change from the same biopsy specimen (arrow). Images were taken at 40× magnification, courtesy of Dr. Susan Davies.

Changes in the response of the liver to injury with age

One of the most important age-related changes in liver function is the demonstration in animal models of a significant decrease in regenerative capacity of the liver [18–20]. In rat studies utilising the partial hepatectomy model, complete hepatic restoration occurs in older animals, but at a slower rate than in younger animals [21]. Liver regeneration in both young and old animals was complete by day 7 after 70% hepatectomy, but at day 1 younger animals had significantly increased liver mass and increased intrahepatic mitotic activity [22].

Newer evidence indicates an active age-related change in the regulation of hepatocyte proliferation. Iakova et al. demonstrated that repression of hepatocyte proliferation after hemi-hepatectomy was switched actively from one intracellular pathway to another in older rat hepatocytes [23]. C/EBPα represses hepatocyte proliferation through inhibition of cyclin dependent kinases (CDK), but after hemi-hepatectomy it undergoes down-regulation to allow liver reconstitution [24]. Iakova et al. demonstrated that aged rat hepatocytes expressed higher levels of the chromatin remodelling protein Brm. Interaction of C/EBPα with Brm led to the formation of high molecular weight complexes leading to switch of proliferative repression from CDKs to E2F-promoted genes [23]. In their model the repression switch was associated with impaired hepatocyte proliferation after hemi-hepatectomy.

Clinical outcome in human acute liver injury is also in part dependent upon the potential for hepatic regeneration. Older people have a higher incidence of acute liver failure and a higher mortality with acute hepatitis A [25,26]. In a cohort of patients with acute liver failure secondary to viral hepatitis, age was an independent predictor of a poor outcome with those over fifty years old faring worst [27].

Ageing and chronic liver diseases

Increasing age has now been recognised as a significant correlate of a poor outcome in a number of chronic liver disorders (Table 1). Perhaps the best studied is chronic HCV infection. Wong et al. and Poynard et al. were among the first to demonstrate that age at infection was a major risk factor for subsequent fibrosis progression [5,28] and further, that the rate of fibrosis progression accelerated with increasing age [29]. Other groups have confirmed these findings; individuals older than 37 at the time of infection

Table 1. Recent studies linking age and clinical outcome in a variety of liver diseases. HCV, hepatitis C virus; HBV, hepatitis B virus; PBC, primary biliary cirrhosis; AIH, autoimmune hepatitis; NAFLD, non-alcoholic fatty liver disease; A1-AT, alpha-1 anti-trypsin deficiency.

Disease	Authors	Year	Summary	Ref
HCV	Poynard et al	1997	Age at infection older than 40 years associated with accelerated fibrosis progression	[5]
	Pradat et al	2006	Age at infection above 37 years independently associated with faster fibrosis progression	[31]
	Ryder et al	2004	Age at biopsy independently associated with fibrosis progression in paired biopsy study	[32]
	Minola et al	2002	Increased age at infection independently associated with risk of development of cirrhosis	[33]
	Wright et al	2003	Increased age at infection and biopsy independently associated with faster fibrosis progression	[34]
	Poynard et al	2001	Increasing age was associated with increased rates of fibrosis progression	[29]
HBV	Poynard et al	2005	Increasing age independently associated with presence of bridging fibrosis	[144]
	Papatheodoridis et al	2005	Increasing age associated with accelerated fibrosis progression in HBeAg —ve HBV	[145]
	Chu et al	2009	HBV reactivation and development of cirrhosis associated with increased age	[146]
PBC	Goudie et al	1989	Increasing age associated with liver-related mortality in PBC	[35]
	Chan et al	2005	Increasing age associated with death in PBC	[147]
	Prince et al	2002	Increasing age independently associated with death in PBC	[148]
Alcohol	Forrest et al	2005	Increased age independently associated with mortality in alcoholic hepatitis	[36]
	Louvet et al	2007	Increased age independently associated with mortality in alcoholic hepatitis	[149]
	Dominguez et al	2008	Increased age independently associated with mortality in alcoholic hepatitis	[150]
AIH	Floreani et al	2006	Higher rate of acute presentation and higher stage of fibrosis in aged patients	[151]
	Al-Chalabi et al	2006	Increased rate of decompensation at presentation in older subjects, but increased rate of response to steroids	[152]
	Czaja et al	2006	Increased rate of cirrhosis at presentation	[153]
NAFLD	Ratziu et al	2000	Age >50 independently associated with presence of septal fibrosis on biopsy	[37]
	Angulo et al	1999	Increased age independently associated with the presence of bridging fibrosis or cirrhosis	[38]
A1-AT	Dawkins et al	2003	Increasing age independently predicted respiratory and all-cause mortality	[154]
Haemochromatosis	Olynyk et al	2005	Increasing age predicted presence of severe fibrosis	[155]
	Stickel et al	2005	Increasing age independently predicted faster progression to cirrhosis	[156]
Budd-Chiari	Garcia-Pagan et al	2008	Post-TIPSS, increasing age predicted lower transplant-free survival	[157]
	Zeitoun et al	1999	Age at diagnosis independently predicted outcome	[158]

have accelerated fibrosis compared to younger individuals [30–34].

Studies of other liver diseases have demonstrated similar findings. Age is an independent risk factor for poor outcome in primary biliary cirrhosis (PBC) in addition to the presence of portal hypertension and impaired liver function [35]. Similarly, age independent of bilirubin, prothrombin time, and renal function predicted outcome in patients with alcoholic hepatitis [36].

There is some evidence that age may influence disease progression in non-alcoholic fatty liver disease (NAFLD). Studies from France and USA have shown that increasing age, increasing body mass index, and type-2 diabetes were associated independently with cirrhosis in patients with NAFLD [37,38]. Being older than 50 years gave an odds ratio of 14 for the presence of at least severe fibrosis [37].

Work by Wali et al. [39] and Berenguer et al. [40] indicate an association between advancing allograft donor age and more rapid histologic progression after HCV graft infection. If the liver donor was younger than 40 years, the median interval to post-transplant cirrhosis was 10 years compared to only 2.2 years when the donor was aged 50 years or more [39]. Berenguer et al. studied 522 patients undergoing liver transplantation between 1991 and 2000, including 283 (54%) infected with HCV. Patient survival was lower in HCV positive recipients and they suggest that the increasing age of donor organs was contributing to decreased patient survival in recent years [40].

Mechanisms of ageing and senescence in the liver

There has been considerable progress in elucidating mechanisms that regulate the capacity for cell division and regeneration in the context of ageing. Senescence is a stress-responsive programme limiting the proliferation of damaged cells and leading to stable cell-cycle arrest [41]. It was initially identified as replication-induced senescence of cultured human fibroblasts by Hayflick et al. [42]. However, it can develop in many cell types in response to a range of cellular stressors such as telomere dysfunction, oxidative, and oncogenic stress (Fig. 3) [43]. This review focuses on the evidence for replicative senescence and telomere signalling in the pathogenesis of liver disease and hepatic neoplasia.

Telomeres and DNA damage signalling

Substantial attention has focussed on the telomere/telomerase system as a mediator of replicative capacity [44]. Telomeres are

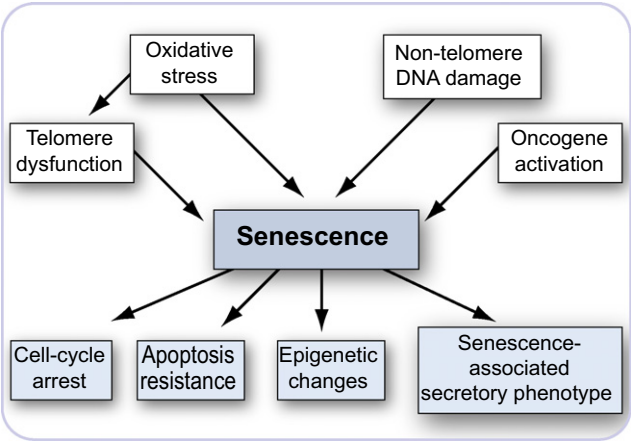

Fig. 3. Causes and consequences of cellular senescence. Cellular senescence can be caused by a number of cellular stresses including telomere dysfunction. Other causes include oxidative stress, non-telomeric DNA damage, and oncogenic activation. Cellular senescence is characterised by cell-cycle arrest, resistance to apoptosis, epigenetic changes including formation of senescence-associated heterochromatic foci, and the senescence-associated secretory phenotype.

repeating hexanucleotide sequences which, with their associated protein complexes, function to protect chromosomes against chromosomal end-end fusion and non-reciprocal translocations.

Telomere length can be measured by a number of experimental methodologies (Fig. 4). Enzymatic digestion of telomere DNA and Southern blotting remains the standard but may soon be superseded by PCR-based assays; these methods give absolute telomere lengths. However, telomere lengths of specific cells or cellular sub-populations are difficult without prior cell sorting. *In situ* hybridisation assays can be performed on cells either in suspension as a flow-based assay (Fig. 4A) or on tissues (Fig. 4D). This has the advantage of allowing telomere analysis of single cells or specific cell populations. However, the telomere length generated is measured as fluorescence intensity relative to an internal control, not an absolute length. An estimate of absolute length can be achieved through reference to a paired sample analysed by Southern blotting.

Telomere structure includes a double-strand component of 10–12 kb of TTAGGG repeats and a 3′ single-strand overhang of several hundred nucleotides. The 3′ overhang inserts in the upstream double strand telomere sequence to form a protective T-loop [45]; this protects the telomere from developing a DNA damage response (Fig. 5A).

The shelterin complex of peri-telomere proteins, including telomere repeat-binding factors 1 and 2 (TRF1 and TRF2) (Fig. 5B), binds to the double-stranded telomere and is crucial for both the structure and function of telomeres. Shelterin is

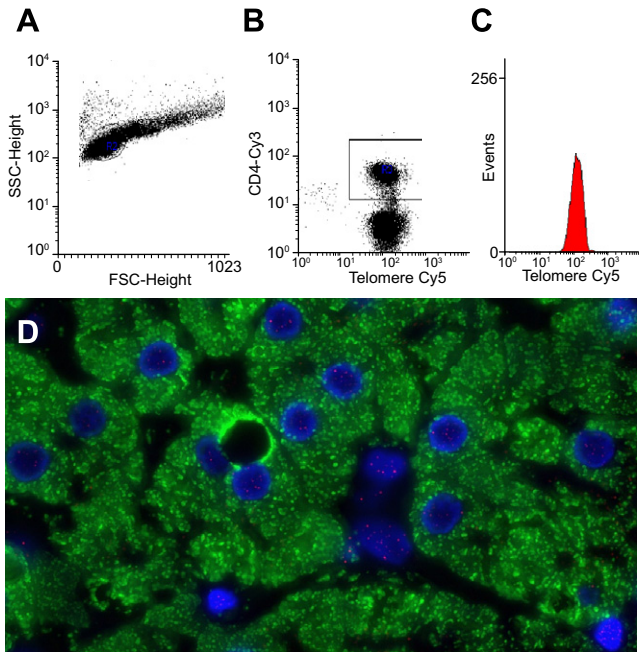

Fig. 4. Telomere analysis by *in situ* hybridisation. (A–C) Examples of experimental methodologies demonstrating telomeres in human CD4+ lymphocytes by flow cytometric FISH, and (D) hepatocytes by quantitative FISH. Fresh human PBMCs are stained for CD4 and then a fluorochrome-labelled telomere consensus sequence is hybridised. Cells are then analysed in a flow-cytometer, gating on live lymphocytes (A), CD4+ lymphocytes (B), and then telomere length can be inferred from the mean fluorescence intensity (C). Paraffin-embedded liver biopsy tissue from a subject with NAFLD was stained with the hepatocyte-specific antibody Hep Par-1 (Green), nuclear stain DAPI (blue), and fluorochrome-labelled telomere consensus sequence (red) allowing study of telomeres in single cells or cellular populations. (Image courtesy of Dr. Aloysius Aravinthan).

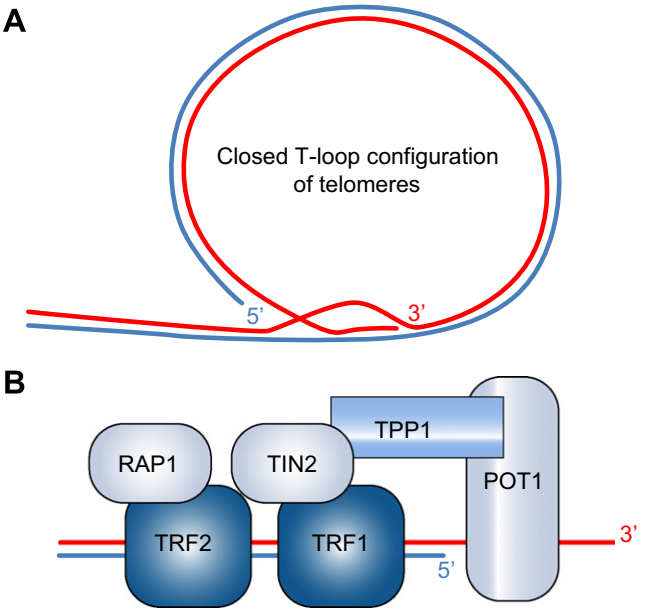

Fig. 5. Structure of mammalian telomeres. (A) The G-rich 3′ single-stranded overhang inserts into the upstream double-stranded telomeric DNA to form a protective t-loop configuration at the end of chromosomes. (B) Peri-telomeric shelterin complex contains six proteins in mammals: TRF1, TRF2, TIN2, RAP1, TPP1, and POT1. POT1 prevents the generation of a single-stranded DNA damage response by binding to the 3′ overhanging strand. Adapted from Cesare and Reddel, 2010 [136].

abundant at the telomere but not elsewhere in nuclear DNA, due to the high-specificity binding sites for telomere repeats in TRF1 and 2, [46]. The importance of TRF2 was demonstrated by van Steensel et al. in a fibrosarcoma cell line [47]. Expression of a dominant negative allele of TRF2 led to end-to-end chromosomal fusions despite the presence of adequate telomere length. Protector of telomeres-1 (POT1) binds to the single-strand DNA overhang to prevent the development of a single-strand DNA damage response and functions with TPP1 to facilitate binding of telomerase [48].

In the absence of compensatory mechanisms, telomere DNA shortens with each division, reflecting incomplete synthesis of telomere termini during chromosomal replication [4]. With repeated cell division a point of critical telomere shortening is reached and a growth arrest signal is expressed, preventing further cell division. Short or dysfunctional telomeres cannot protect chromosomal integrity from progressive attrition or uncapping associated with loss of shelterin complex components such as TRF2 [49].

Short telomeres are detected as double-strand DNA breaks by the MRN complex of proteins, including MRE11, NBS1, and RAD50 (Fig. 6) [49,50]. With progressive loss of telomeres, there is a commensurate loss of inhibitors of DNA damage response elements such as TRF2 [50]. Loss of this inhibition leads to recruitment of the PI3-kinases ATM and ATR, phosphorylation of Histone 2A at serine 139 (γ-H2AX) and stabilisation of p53 [4,50,51], and p21 [52,53]; the involvement of p16 in the induction of senescence is controversial [43,53]. The mechanisms that determine whether a cell undergoes senescence or apoptosis in response to telomere dysfunction-induced-DNA damage response are not understood. Replicative senescence is characterised *in vitro* by growth arrest,

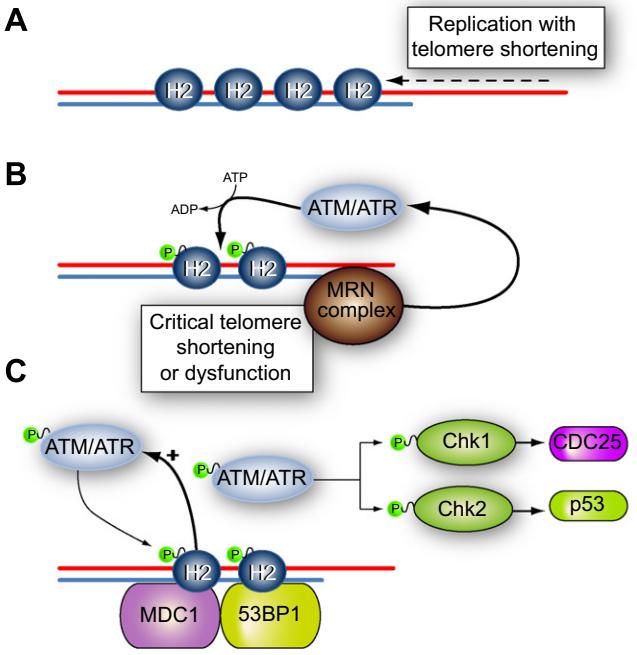

Fig. 6. Short telomeres generate a double strand break (DSB) DNA damage response. Progressive telomere shortening is signalled to ATM/ATR by the MRN complex leading to phosphorylation of Histone 2 (H2) at serine 139 to form γ-H2AX (B). Generation of γ-H2AX leads to a positive feedback loop-inducing phosphorylation of further ATM/ATR. γ-H2AX also allows binding of other factors in the DSB response pathway including the mediator of DNA damage checkpoint protein 1 (MDC1) and p53 binding protein-1 (53BP1) [50] (C). Activation of ATM/ATR leads to downstream signalling through phosphorylation of the checkpoint kinases CHK1 and CHK2 leading to activation of p53 and arrest of further mitosis.

an inability to respond to external mitogens, and altered cell morphology. Short telomeres detected during DNA replication at mitotic S-phase may induce apoptosis [54].

Under certain circumstances telomeres can form a four-strand quadruplex rather than T-loop structure which prevents access of telomerase to the 3′ overhanging strand. This is associated with accelerated telomere attrition leading to senescence [55].

Accelerated telomere shortening has been demonstrated in conditions associated with inflammation and accelerated cell turnover [56], leading to the concern that continued hepatocyte turnover in chronic inflammatory liver disease may lead to telomere-mediated loss of replicative ability and a reduced potential for regeneration. Further, known risk factors for ill-health (and which play a part in chronic liver disease) such as obesity and smoking are associated with accelerated loss of telomere DNA [57,58].

Several studies have investigated telomere length and downstream telomere signalling in healthy hepatocytes with increasing age. Wang et al. studied mice of increasing age and demonstrated *in vivo* accumulation of γ-H2AX foci within hepatocytes [59]. However, mice have telomeres that are significantly longer than those in humans and there was no significant co-localisation between γ-H2AX foci and telomeres in gut epithelium. This suggests that the DNA damage response may rely more on accumulation of DNA damage distant to the telomere with advancing age. Aikata et al. demonstrated that telomere length in human liver, without reference to cell type, measured by restriction fragment length declined with age by around 120 base pairs per year, but that no normal liver sample had telomere lengths shorter than 5 kb [60]. Other groups have demonstrated a decline in hepatic telomeres from 9.7 kb at age 20 to 8.1 kb at age 60, a decline of 40 bp per year [61].

Telomerase

Telomerase is a reverse transcriptase consisting of enzymatic (TERT), RNA template (TERC) and several other protein components including heat-shock protein 90 (hsp90) and dyskerin [62]. Telomerase can maintain telomere length by adding TTAGGG repeats, but its expression is controlled in differentiated [63,64] and stem-cell populations [65]. In stem-cell populations over-expression of telomerase activity led to increased proliferation of epidermal stem cells and differentiation of their progeny [65]. Ectopic expression of telomerase in retinal pigment epithelial cells and fibroblasts led to lengthening of telomeres, continued cell division, and extension of *in vitro* lifespan [66]. Re-expression in non-stem-cell populations in healthy life is unusual, but telomerase is expressed in around 80% of human carcinomas, including hepatocellular carcinoma [67].

A recent genome wide-association study looking at the natural variability of telomere length in healthy human populations has identified single-nucleotide polymorphisms on chromosome 3q, an area coding TERC, which was associated with shorter telomeres. Each copy of the identified allele was associated with telomere shortening equivalent to around 4-years natural ageing [68].

Recent evidence has suggested that telomerase may have a role in several cellular pathways beyond its known role in elongation of telomeres and replicative ageing. Regulation of apoptosis, gene expression, and chromatin structure during the cell-cycle has been ascribed to telomerase activity [69].

Genetic evidence for a role of telomere dysfunction in human ageing comes from the discovery that germ-line mutations of the telomerase complex component dyskerin cause the Progeroid syndrome Dyskeratosis Congenita (DKC) [3,70]. Patients with DKC are at increased risk of malignancies, pulmonary fibrosis, and cirrhosis. However, unchecked telomere shortening leads eventually to bone marrow failure, which is fatal often in the second or third decade.

In the field of liver disease, there is a growing body of evidence for the role of hepatocyte telomere shortening in relation to clinical outcome. Reduction in whole liver or hepatocyte telomeres occurs with normal ageing and may give an indication of residual proliferative potential [60,71]. Aikata et al. demonstrated a progressive decline in telomere length in whole liver biopsy samples with increasing age in both healthy subjects and subjects with chronic liver disease [60]. Takubo et al. demonstrated that telomere length in whole liver biopsy specimens declines at a similar rate to that in highly proliferative tissues such as digestive tract mucosa [71].

Telomeres in chronic liver disease

Short human telomeres have been linked to the subsequent onset of disease and death [72] in healthy older persons. Both Kitada et al. and Urabe et al. showed that subjects with chronic viral hepatitis had shorter hepatic telomeres than healthy controls and that increasing fibrosis was associated with shorter telomere

lengths [73,74]. However, their methodology was based on homogenates of liver tissue with restriction fragment length analysis, which precluded identification of the particular liver cells that underwent telomere shortening. Wiemann et al. demonstrated that hepatic telomere length was shortened in cirrhosis irrespective of the primary disease aetiology and suggested that the shortening was restricted to hepatocytes. Using a fluorescence *in situ* hybridisation method, they demonstrated telomere shortening and senescence-associated β-galactosidase (SA-βGAL) expression in hepatocytes but not in stellate cells or intrahepatic lymphocytes [61]. Sekoguchi et al. confirmed the negative association between hepatic fibrosis and telomere length in liver biopsy specimens from HCV-infected subjects utilising *in situ* hybridisation to confirm the relationship in hepatocytes [75]. Further, they demonstrated a significant association between the rate of telomere shortening and the rate of fibrosis progression.

Ikeda et al. found that large cell change, frequently found in liver biopsy specimens from older subjects (Fig. 2D), represented senescent hepatocytes in a study of human liver tissue from patients with chronic viral hepatitis [76]. SA-βGAL and other markers associated with senescence such as p21 were expressed strongly in areas of large cell change.

Rudolph et al. demonstrated the importance of the telomere system in chronic liver disease in a mouse model; shortened hepatocyte telomeres induced through telomerase knockout were associated with an accelerated onset of cirrhosis in the context of chronic liver injury induced with carbon tetrachloride [77]. Reintroduction of telomerase activity with an adenoviral vector prevented critical telomere shortening, the onset of cirrhosis and deterioration of liver function [77].

Similarly, Wiemann et al. utilised a late generation telomerase knockout model to demonstrate that mice with short hepatocyte telomeres had decreased survival when subjected to chronic liver injury [78]; mice with short telomeres (induced through telomerase knockout) and chronic liver damage (induced by hepatocyte specific expression of HBV surface antigen under an albumin promoter) had reduced survival when compared to litter-mates with longer telomeres [78]. Notably however, mice with short telomeres showed strong inhibition of HCC formation emphasising the dual role of telomere shortening.

The role of p53 in healthy ageing and cancer

An age-related induction of replicative senescence at the cellular level, mediated by the telomere system, may be the price of continued tumour suppression for the whole organism [79,80]. In mice, driven to senescence by induction of critically short telomeres in a telomerase knockout model [81], dysfunction of multiple organs develops with impairment of tissues requiring high levels of cell replication such as skin, bone marrow, and reproductive organs [81]. Cells demonstrated impaired proliferation and increased rates of apoptosis but interestingly animals did not accumulate senescent cells [52]. However, cells from these mice demonstrated multiple features of telomere-dysfunction with aneuploidy, chromosomal end-to-end fusions, and high p53 expression.

These features of cellular and organ senescence can be reversed through a subsequent lesion of p53, thereby preventing telomere dysfunction to signal through to cell-cycle arrest, allowing further *in vitro* cell division and *in vivo* preservation of organ function [82]. Telomere shortening in the telomerase deficient mouse line was associated with increased expression of p53, growth arrest, and increased apoptosis. Simultaneous lesions of telomerase and p53 prevented testicular atrophy and cells had reduced levels of apoptosis with higher rates of passage through the cell cycle [82]. However, other studies studying organ specific deletion of p53 in the context of telomerase-deficient mice, suggest that intestine-specific loss of p53 allows the persistence of cells with chromosome instability. These cells gave rise to apoptosis-prone intestinal epithelium and therefore premature intestinal failure [83].

However, while these mice have a partial reversal of their aged phenotype, there was an increased risk of *in vitro* cellular transformation and *in vivo* development of skin, breast, and gastrointestinal carcinoma [84]. In contrast to telomerase-deficient mice, compound telomerase and p53 deficient mice have a high rate of development of epithelial carcinomas which are associated with complex non-reciprocal translocations as a consequence of loss of telomere protection of chromosomal ends. Therefore, these data suggest that telomere attrition in tissues with a high turnover rate leads to the development of a DNA damage signal through p53 and subsequent senescence before loss of telomere chromosomal protection can lead to the development of carcinogenic genetic translocations [84].

More recent studies on a similar mouse model by Choudury et al. have shed further light on the downstream mechanisms of telomere dysfunction [52]. In late generation telomerase-deficient mice, a subsequent lesion of the cell-cycle inhibitor p21 prevented the accelerated ageing phenotype and organ dysfunction associated with dysfunctional telomeres but did not lead to an increase in chromosomal instability or increase in tumour formation demonstrated in the p53 deficient mouse. These two studies suggest that p21 may mediate cellular senescence downstream of dysfunctional telomeres and that p53 has a wider role in tumour suppression and prevention of cellular transformation.

This interpretation has been reinforced by a study by Xue et al. [85]. They utilised a tetracycline responsive siRNA to repress p53 in a mouse model of hepatoblastoma [85]. Re-expression of p53 led to rapid involution of the tumours due to the development of cellular senescence rather than apoptosis. Further, they demonstrated that the innate immune system was important in the clearance of senescent cells; antibody or toxin mediated suppression of neutrophil, macrophage or NK cell function prevented tumour regression [85].

Telomeres in HCC

Intact telomere signalling has also been demonstrated to be important in the development of hepatocellular carcinoma (HCC). Kojima et al. demonstrated that around 85% of human HCC specimens show reactivation of telomerase activity [86], providing a selection advantage aiding uncontrolled hepatocyte replication.

The beneficial effects of the intact telomere/telomerase system have been demonstrated with suppression of the development of HCC in a mouse model [78]. Utilising a telomerase knock-out mouse model, Wiemann et al. demonstrated that short hepatocyte telomeres were associated with marked inhibition of HCC formation in a late generation telomerase knock-out mouse model [78]. Similarly Lechel et al. demonstrated that telomerase

knockout was associated with HCC suppression in a mouse model of chronic liver injury [87]. The development of spontaneous replicative senescence in HCC-derived Huh7 cells is associated with repression of telomerase, telomere shortening, and cell-cycle arrest [88].

Kim et al. investigated the role of telomere length changes and downstream telomere signalling in dysplasia and neoplasia in liver biopsies from chronic hepatitis B virus infected subjects [89]. With progression from normal hepatocytes through large cell change, small cell change, and then neoplasia there was a progressive decline in hepatocyte telomere length measured by *in situ* hybridisation and evidence of downstream activation of the double-strand DNA break pathway with increased phosphorylation of H2AX [89]. Telomeres within HCC were shorter than surrounding non-cancerous liver [90], suggesting that HCC had escaped the DNA damage response and subsequent cell-cycle arrest signal generated from short telomeres. Other studies of HBV-associated HCC have demonstrated longer telomeres and higher telomerase activity in hepatocytes from HCC and that these features correlate with a worse prognosis [91]. Oh et al. demonstrated increasing transcriptional expression of shelterin components from normal to dysplastic to neoplastic hepatocytes in human liver [92]. Further, they demonstrated declining telomere lengths with neoplastic hepatocytes having the shortest telomere length. Lee et al. have extended these finding in HBV-associated carcinogenesis identifying an increase in micronuclei formation, p21 inactivation and reduction in telomere length from low grade dysplastic nodules to high grade dysplastic nodules to HCC [93].

Interestingly, short telomeres or telomere dysfunction appears permissive for the development of early stage neoplasia, but inhibitory to later stage and more anaplastic lesions [94].

The role of the telomere/telomerase system in the pathogenesis of HCC have led some to suggest that therapeutic manipulation may hold the promise for future therapies [63]. Telomerase inhibitors are currently in phase II clinical trials and would represent a rational therapy for HCC. However, the potential for inducing fatal liver failure, given the results from mouse models, may limit their safety and efficacy.

Oxidative stress

Senescence can occur with oxidative stress or DNA damage distant to the telomere [43,95]. Zhang et al. [96] compared hepatocyte injury in response to hypothermic challenge, leading to increased oxidative stress, in young and old rats and concluded that old rats were more susceptible. There is evidence for both a decline in DNA repair and an increase in DNA or cell sensitivity to oxidative stress with increasing age. Intano [97] reported a 50% age-related decline in DNA base excision repair in old age; Hamilton [98] attributed the increased levels of oxidative damage suffered by DNA in senescent mice and rat livers to increased DNA or cell sensitivity to oxidative stress. How cell sensitivity to oxidative stress is mediated is unclear. This may be mediated by NF-κB [99] which has increased activity with ageing and which may induce target genes including haemoxygenase. Up-regulation of pro-apoptotic genes, such as Gadd153, which increase cellular sensitivity to oxidative stress, may also play a role [100].

Oxidative stress and telomeres

Recent evidence suggests that oxidative stress accelerates telomere shortening [101,102]. It has long been known that low ambient oxygen conditions can extend the lifespan of cells in culture [103]; however the underlying mechanism of this effect was uncertain. Studies have demonstrated that when cultured cells are protected from oxidative stress through low ambient oxygen tension, addition of anti-oxidants or over-expression of antioxidant enzymes, telomere shortening is slowed [104–106].

Telomeres are highly susceptible to oxidative-induced-DNA damage because of their high guanine content [107]; a human telomere sequence inserted into a plasmid suffered seven times more DNA damage due to hydrogen peroxide-induced oxidative damage than a control DNA sequence [108]. Reactive oxygen species cause single-strand DNA breaks and telomeres lack the surveillance mechanisms for single-strand breaks that are employed in the rest of the genetic material [109]. Induced single-strand breaks in non-telomere DNA were repaired within one day, whereas DNA repair within the telomere was delayed and ultimately incomplete [109]. The mechanism linking accumulation of oxidative DNA damage and accelerated telomere shortening is uncertain [101]. One possibility is the extrusion of telomerase from the nucleus of cells undergoing oxidative stress, thus preventing the usual intra-nuclear role of telomere preservation. Once in the cytoplasm, telomerase co-localises with mitochondria [110]. In cells with over-expression of telomerase, mitochondrial DNA is protected from oxidative damage and cellular levels of reactive oxygen species are lower than control cells [110].

Further data has demonstrated the important interaction between telomere-induced senescence and oxidative stress. Passos et al. demonstrated that cells driven to either irradiation- or telomere-induced senescence developed increased mitochondrial ROS production [111]. Further, they demonstrated that knockdown of either p53 or p21 prior to induction of senescence prevented the increased mitochondrial ROS production. They demonstrated that activation of p21, or downstream signalling through TGF-β, was able to induce further DNA damage foci and the subsequent DNA damage response, leading to the conclusion of a positive feedback loop [111]. Senescence leads to the development of oxidative stress which reinforces the senescent state of the cell and causes further oxidative stress.

Whether the combination of telomere shortening and oxidative stress represents the double hit required to accelerate progression of liver diseases is uncertain. Certainly the combination of two concurrent liver diseases leads to accelerated disease progression and a worse outcome. For example, both diabetes mellitus (DM) and alcohol misuse accelerate fibrosis progression in chronic HCV infection [5,30,112,113]. It has been described that HCV is associated with shortened intrahepatic telomeres [61] and both DM [114] and alcohol [115] are known to cause oxidative stress through mitochondrial dysfunction. In their study of hepatocyte telomeres Sekoguchi et al. investigated the relationship between hepatocyte telomeres, markers of cell-turnover and oxidative stress in chronic HCV-infected patients [75]. They found that hepatocyte telomeres shortened progressively with increasing hepatocyte fibrosis stage and with increasing evidence of hepatocyte oxidative stress as measured by 8 deoxy-guanosine [75].

Viruses and the telomere/telomerase system

A number of viruses causing human disease have evolved mechanisms for manipulating telomere signalling or telomerase to promote replication and transmission [116]. EBV and HPV encode proteins that up-regulate telomerase activity, which may play a role in the human cancers associated with these viruses.

HBV is associated with a high incidence of HCC. Several groups have reported a role of HBV genome integration and up-regulation of telomerase activity. In particular the HBV X protein disrupts p53 signalling as well as other cellular signalling pathways including protein kinase C and NF-kappa B [117]. Several groups have demonstrated a direct role of HBV X protein in the up-regulation of telomerase activity [118,119]. Others have demonstrated that other HBV associated proteins also up-regulate telomerase activity [120]. This ability to bypass functional telomere control of replication might predispose to HCC formation. However, some groups have demonstrated *suppression* of telomerase activity [121]. Recent data have demonstrated a similar effect of HCV proteins upon telomerase activity. Zhu et al. demonstrated a twofold increase in telomerase activity in Huh7 cells expressing HCV core protein compared to mock transfected cells [122]. There were detectable increases in telomerase mRNA, protein, and promoter activity; these changes were associated with significantly longer telomere length in cells expressing HCV core, suggesting functional telomerase activity.

Downstream signalling from short telomeres can lead ultimately to cell-cycle arrest. Recent data suggest that cell-cycle arrest may be advantageous to different viruses. In a p53-knockout fibroblast line, CMV replication was noted to be significantly less efficient when p53 was not present [123], suggesting that some viruses induce a state of cell-cycle arrest to aid replication and spread. This effect has also been demonstrated for hepatitis B virus replication. Huang et al. demonstrated that four differing methods of inducing cell-cycle arrest in the host cell line all led to increased HBV replication [124]. Induction of G1 cell-cycle arrest in HepG2 cells led to a reduction in telomerase activity, but a dramatic increase in HBV replication as measured by HBV DNA, HBsAg, and HBeAg in culture supernatants [124].

Tarakanova et al. have demonstrated that murine gamma-herpes virus can subvert the double-strand DNA break pathway to promote viral replication [125]. A virus expressed kinase (orf36) and the homologous EBV kinase (BGLF4) induced phosphorylation of H2AX, leading to both replicative arrest of the host cell and enhanced viral replication. Herpes viral loads were three logs lower when mice were infected with an orf36-mutated virus when compared to wild type virus [125].

Bypassing the telomere system?

Whether all cell types are affected equally by telomere shortening and telomerase activity is not known. Data from the mouse suggested that hepatocytes may bypass the growth inhibitory effects of short or dysfunctional telomeres in certain circumstances [126]. Denchi et al., using a conditional knockout of TRF2 in hepatocytes, demonstrated evidence of telomere dysfunction with telomere fusions and increased γ-H2AX expression. However, despite telomere dysfunction, there was no increase in p53 expression and no reduction in hepatic regeneration following partial hepatectomy, but cell replication did not occur. Instead, the liver was reconstituted by an increase in the size and ploidy of the remaining hepatocytes [126]. Whether similar mechanisms operate within the human liver is unknown. Other groups have demonstrated that human hepatocyte replication is limited by telomere shortening [127].

Other markers in senescence

The presence of senescence can be inferred by other markers. In particular senescence associated β-galactosidase (SA-βGAL) is associated with replicative senescence. In a study of liver tissue from subjects with chronic HCV infection, the presence of SA-βGAL positive cells was associated with increasing age and increasing levels of fibrosis [128]. Further, analysing the role of senescent cells in donor allografts has demonstrated a significant relationship between SA-βGAL positive cells on the reperfusion biopsy and the subsequent rate of fibrosis progression of post-transplant HCV recurrence [129].

Senescence of other cell types within the liver

The evidence that hepatocytes may be more resistant to the effects of replicative ageing and telomere shortening is intriguing [126]. The evidence linking chronic liver disease with telomere shortening and the rescue of experimentally induced cirrhosis by telomerase gene delivery [77] generates the possibility that senescence affects cell types within the liver other than hepatocytes.

A recent study by Krizhanovsky et al. demonstrated the crucial role of stellate cell senescence in hepatic fibrosis progression in a murine model subjected to CCl_4-induced liver injury [130]; there was accumulation of stellate cells bearing markers of senescence such as SA-βGAL, with a reduced capacity to synthesise extracellular matrix components. In mice with knockouts of senescence-associated genes, stellate cells failed to develop senescence and therefore developed enhanced hepatic fibrosis. It is perhaps counter-intuitive to find that fibrosis progression was associated with a failure of stellate cells to undergo senescence when cirrhosis is associated with hepatocyte senescence. Previous human studies have highlighted hepatocyte rather than stellate cell senescence in the context of chronic liver injury so the relevance of this model to human cirrhosis must be questioned.

Sasaki et al. investigated the role of telomere length changes in biliary epithelium in Primary Biliary Cirrhosis (PBC) [131]. Chronic liver disease relates to chronic inflammation of bile ducts. They demonstrated short telomeres, increased SA-βGAL, γ-H2AX, and increased expression of p16 and p21 in biliary epithelial cells from subjects with PBC [131].

Our group has recently published data examining the association between changes in lymphocyte telomere length and clinical outcomes in chronic HCV infection [132]. Subjects with shorter telomere length in the general CD4+ T-lymphocyte population had higher fibrosis stage, poorer survival, and reduced likelihood of responding to anti-viral therapies [132].

The precise interplay of differential ageing of various intrahepatic cell types in different hepatic diseases may be far more complex than first imagined.

Form and function of senescent cells

There is an increasing body of work demonstrating the role of senescence *in vivo*. With the onset of cellular senescence cells can remain viable within tissues for long periods; resistance to apoptosis is a characteristic of senescent cells [41,128]. These cells have a significant change in their form and function.

Induction of senescence of fibroblasts *in vitro* leads to a change to larger, flatter cells with increasing irregularity of shape [4]. An increasing body of work describes the change in the cellular secretosome. This senescence-associated secretory phenotype (SASP) has been demonstrated in other systems to be pro-inflammatory (Fig. 7). Kuilman et al. demonstrated that senescent cells secrete large quantities of IL-6 among other factors such as IL-8 [133]. They demonstrated that IL-6 acted in a paracrine manner to cause other cells to develop senescence. Depletion of IL-6 prevented this 'infectious senescence' [133].

This raises the possibility that once hepatocyte senescence develops, the change in the tissue micro-environment wrought by the SASP could lead to other hepatocytes becoming senescent in a positive feedback loop.

Senescence and the liver – potential therapeutic options

The confluence of senescence, oxidative stress, ageing, and fibrosis progression suggests possible mechanisms by which fibrogenesis and clinical outcome may be manipulated. Avoidance of factors which are associated with oxidative stress and telomere dysfunction should be recommended in line with current hepatology practice, which include recommendations to limit alcohol intake [115], control blood glucose [114], stop smoking, and reduce weight [57,58].

Future interventions to manipulate telomere length or function and downstream p53 signalling in ageing and cancer will require careful study. If we adopt a strategy to attempt to elongate telomeres, by for example inducing telomerase activity to improve hepatocyte proliferation and thence liver function in chronic liver disease, there is a substantial risk of cellular transformation and carcinogenesis. However, the effect of expression

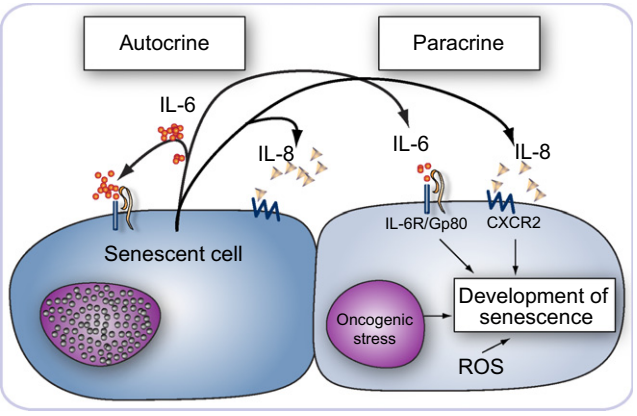

Fig. 7. Senescent cells develop the senescent-associated secretory phenotype (SASP). Senescent cells secrete large quantities of IL-6, IL-8, and plasminogen activator inhibitor-1 [133,137]. These can act in both an autocrine or paracrine manner to reinforce or induce senescence within cells. IL-6 acting via IL-6R/GP80 [133] or IL-8 acting via CXCR2 [137] act in concert with oncogenic stress to induce the senescent phenotype. ROS, reactive oxygen species.

Key Points

- Age is an important prognostic factor in a wide range of liver diseases.
- Telomeres shorten with age, but this decline can be accelerated by oxidative stress and chronic inflammation.
- Hepatocyte telomeres are short in a range of liver diseases and shortening carries prognostic information.
- The intact telomere / telomerase system is a powerful tumor suppressor mechanism acting through p53.
- Inactivation of telomerase and evidence of chromosomal instability occurs in the majority of HCC.
- Senescence and oxidative stress act in a positive feedback loop.
- A number of viruses have evolved mechanisms to subvert the telomere / telomerase system to promote viral replication and infectivity.

of telomerase could have several effects. In cells with pre-existing damage to cell cycle check-points telomerase activity could be permissive of unchecked replication, full cellular transformation, and oncogenesis. However, in cells with intact cell cycle check-points, elongation, and stabilisation of telomeres could reduce selection pressure for outgrowth of mutant hepatocyte clones with damaged check-points, ultimately reducing cancer carcinogenesis.

However, on the other hand, utilising telomerase inhibition therapy in the context of HCC [134,135] runs the risk of impairing remaining hepatocyte proliferative ability and possibly leading to hepatic decompensation. Wiemann et al. demonstrated in late generation telomerase knockout mice that short telomeres strongly inhibited HCC formation, but survival was reduced due to impairment of hepatocyte regeneration [78].

However, early studies using small molecule inhibitors or RNA interference have demonstrated that telomerase inhibition may be a useful therapeutic strategy in the context of HCC [134,135]. Further, as we discover that telomerase has more activities than simply elongating telomeres [110], can we deploy therapeutics which may impair the cellular defences against oxidative damage in the context of pro-oxidant diseases such as hepatitis C- or alcohol-related liver disease?

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript

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