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Review

Retinoids in health and disease: A role for hepatic stellate cells in affecting retinoid levels

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ABSTRACT

Vitamin A (retinol) is important for normal growth, vision and reproduction. It has a role in the immune response and the development of metabolic syndrome. Most of the retinol present in the body is stored as retinyl esters within lipid droplets in hepatic stellate cells (HSCs). In case of liver damage, HSCs release large amounts of stored retinol, which is partially converted to retinoic acid (RA). This surge of RA can mediate the immune response and enhance the regeneration of the liver. If the damage persists activated HSCs change into myofibroblast-like cells producing extracellular matrix, which increases the chance of tumorigenesis to occur. RA has been shown to decrease proliferation and metastasis of hepatocellular carcinoma. The levels of RA and RA signaling are influenced by the possibility to esterify retinol towards retinyl esters. This suggests a complex regulation between different retinoids, with an important regulatory role for HSCs.

1. Vitamin A

1.1. Family and structure

The ether-soluble substance described by McCollum and Davis [1] that proved vital to the growth of rats after prolonged deprivation, was later called vitamin A or retinol (ROH). ROH can be derived from carotenoids that have an unsubstituted β-ionone ring for conversion to ROH (*via* retinaldehyde) [2], like β-carotene, α-carotene, γ-carotene and β-cryptoxanthin. Vitamin A and its derivatives are collectively called retinoids. Retinoids are isoprenoids, containing a six-membered carbocyclic ring and a sidechain with eleven carbon atoms. Depending on reduction, oxidation, esterification or changes in *cis* or *trans* orientation, the function and activity of the retinoid molecule can change. The storage form of vitamin A is retinyl-ester. Retinaldehyde (Ral) and retinoic acid (RA) are the main biological active molecules. Multiple variants of RA and Ral exist; 9-*cis*-retinoic acid and all-*trans*-retinoic acid (ATRA), and retinaldehyde *i.e.* 11-*cis* Ral, and all-*trans*-Ral, respectively (see Fig. 1) (reviewed by [3,4]).

In this review we will first describe the nuclear receptors that are activated by the various retinoic acids and the major functions of retinoids within the body, then give a brief overview of the uptake and transport of vitamin A, and finally discuss the storage and release of vitamin A in special cells in the liver, the hepatic stellate cells (HSCs), during health and disease.

1.2. Receptors

The biological effects of RA are mainly mediated by two types of nuclear receptors acting as transcription factors. Retinoic Acid Receptor (RAR) binds ATRA and Retinoid X Receptor (RXR) was initially reported to have ligand specificity for 9-*cis*-RA [5]. More recently it has been questioned whether endogenous levels of 9-*cis*-RA are high enough to be physiologically relevant as a RXR ligand. Several other

Abbreviations: ASCL4, acyl CoA synthetase type 4; ATGL, adipose triglyceride lipase; ATRA, all-*trans*-retinoic acid; BCO1, β-carotene-15,15′-monooxygenase; CD36, Cluster Determinant 36; CM, chylomicron; CRABP2, cellular RA binding protein 2; CRBP1, Cellular Retinol-Binding Protein 1; CRBP2, Cellular Retinol-Binding Protein 2; CYP26A1, Cytochrome P450 Family 26 Subfamily A Member 1; DC, dendritic cell; DGAT1, Diacylglycerol O-Acyltransferase 1; FABP5, fatty acid binding protein; HCC, hepatocellular carcinoma; HSC, hepatic stellate cell; HSD17B13, 17-β hydroxysteroid dehydrogenase 13; JAK/STAT, Janus kinase/signal transducers and activators of transcription; LD, lipid droplet; LRAT, lecithin:retinol acyltransferase; NK cell, natural killer cell; NPLC1L1, NPC1-like transporter 1; PNPLA3, patatin-like phospholipase domain-containing 3; PUFAs, poly-unsaturated fatty acids; RA, retinoic acid; Ral, retinaldehyde; RAE1, retinoic acid early inducible gene 1; RAR, retinoic acid receptor; RARE, retinoic acid response elements; RBP4, retinol binding protein 4; RBPR2, retinol binding protein receptor 2; ROH, retinol; RRD, retinal reductase; RXR, retinoid X receptor; STRA6, receptor stimulated by retinoic acid 6; SR-BI, Scavenger Receptor class B type I; TAG, triacylglycerol; TTR, transthyretin

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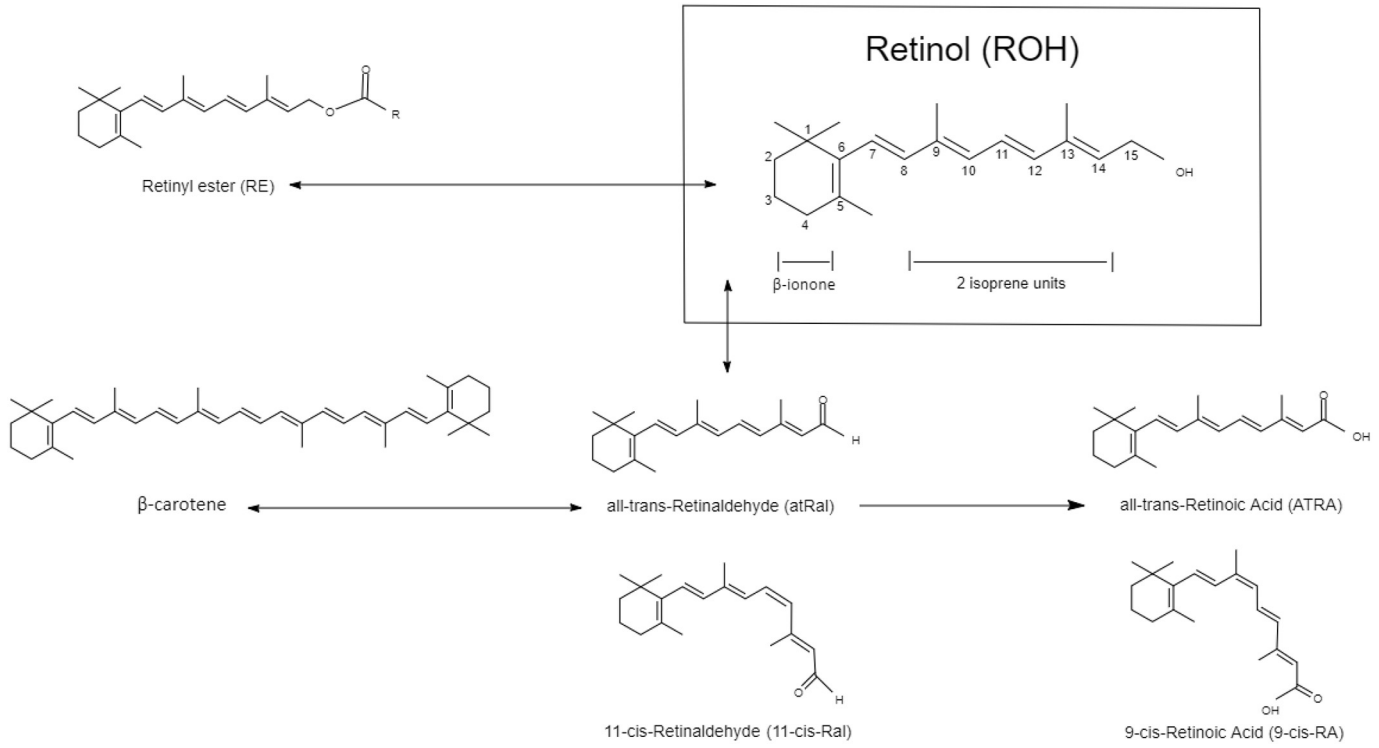

Fig. 1. Nomenclature and structure of the main types of retinoids. 11-*cis*-Retinaldehyde and 9-*cis*-retinoic acid are not connected to the other retinoids by arrows because their formation requires several steps and/or involve different pathways. Together with the two oxygens, R represents the fatty acid moiety within retinyl ester.

candidates have been suggested, among which 9-*cis*-13,14-dihydroretinoic acid [6,7]. Both RAR and RXR have several isotypes, with different tissue distributions (reviewed by [8,9]). RAR and RXRα can form heterodimers, which interact with retinoic acid response elements (RAREs) [8]. RAR and RXR can also form homodimers and RXR has been shown to form heterodimers with a large number of other nuclear receptors [8–10]. These nuclear receptors and their different heterodimers are at the basis of many of the effects caused by RA, but RA and retinol also have a number of extranuclear and nontranscriptional effects (reviewed by [11,12]). One of these non-genomic effects, *i.e.* the effect of retinol on JAK/STAT signaling through STRA6, will also be mentioned below.

1.3. Function

Already in 1913 it has been described that long term deprivation of vitamin A prevents normal growth [1]. Later the role of vitamin A in vision was discovered, more specifically in the detection of light in the retina (reviewed by [13–15]). This also let vitamin A and its derivatives to be named after the retina. In the eye, retinol is converted to 11-*cis*-retinaldehyde, providing the chromophore in the opsins in the retina. When a photon reaches the 11-*cis*-retinaldehyde, it is converted to all-*trans*-retinaldehyde, thereby inducing opsin to change into its active form (reviewed by [16]).

1.3.1. Immune system

Vitamin A is also involved in the defense against pathogens in multiple ways (reviewed by [17,18]). In 1925 Wolbach and Howe noted that rats kept on a vitamin A-deficient diet showed changes in epithelium at several places, including the respiratory tract, the eyes and the genitourinary tract [19]. Years later, the effects of hypo and hypervitaminoses A on epithelium were mimicked in a cell culture system, showing similar effects of para and hyperkeratosis depending on the amounts of retinoic acid in the medium [20]. Vitamin A deficiency also caused a change in the mucous produced by goblet cells [21]. Both of these changes impair the mucosal epithelial barrier of the respiratory and intestinal tract, providing an easier access for pathogens. This is also supported by the fact that a vitamin A-deficient diet let to an increase in the amount of bacteria present in the gastro-intestinal tract of rats and a change in the type of bacteria present [21].

Next to influencing the barrier function of the epithelium, vitamin A is also involved in the differentiation of cells of the innate immune system like macrophages [22], neutrophils [23] and natural killer cells [24,25]. All-*trans*-RA is involved in differentiating precursors of dendritic cells (DC) towards intestinal DCs, known for their anti-inflammatory characteristics [26]. Some studies also suggest this is dependent on whether IL-15 is present or not [27]. A population of CD103+ mesenteric lymph node DCs has been shown to induce the development of Foxp3+ T reg cells, in the presence of TGF-β and RA. The RA was suggested to be produced by the DCs from retinaldehyde [28]. In this way, RA would be involved in creating the intestinal homeostasis where inflammatory responses against the commensal flora and dietary antigen must be suppressed. In contrast to these anti-inflammatory characteristics, RA can influence tissue tropism and subsequently pathogenicity of Th17 cells depending on the concentration of RA [29]. Less literature exists on the effects of RA on B cells. Secretion of IgA by B cells is reduced after RARα knockdown and the exogenous addition of 9-*cis*-RA reduces IgE production and increases IgA production, modulating the type 1 allergic immune response [30]. In summary, RA has a role in regulating the location and severity of an immune response, depending on concentrations and type of RA present. Whereas RA influences the immune response, infectious diseases also influence the retinol levels in the blood. This can be through reduction in uptake, or by an increased loss, for example through loss of retinol binding protein with the urine [17,18,31,32].

1.3.2. Reproduction

Apart from growth, vision and immunity, vitamin A deprivation also

affects the reproduction system. Wolbach and Howe already described edema and atrophy of the testes and keratinizing epithelium in the epididymis, prostate and seminal vesicles, and in the uterus and oviducts after rats were kept on a vitamin A-deficient diet. Both female and male rats became infertile under these conditions [19]. In male mice and rats spermatogenesis is arrested at the spermatogonia stage [33,34]. In the females, next to the keratinized epithelium described by Wolbach and Howe [19], effects of a lack of vitamin A are found throughout the pregnancy. In a diet completely devoid of vitamin A females are able to ovulate and form corpora lutea, but blastogenesis never happens [35]. When low levels of vitamin A are provided, fertilization and implantation occur. During pregnancy, the demand of vitamin A increases, preventing resorption of the fetus or necrosis of the placenta. A lack of vitamin A provided through the diet can cause a range of defects in the fetus (reviewed by [36]).

Within male animals, RA is thought to be produced from retinol by Sertoli cells [36]. In earlier studies, supplementation with RA or retinol in vitamin A deficient rats produced different results [37–39]. More recently, consensus was reached that ATRA is necessary for sperm differentiation (reviewed by [36,40,41]). Even so, it appears that not only RA influences the testes. Male mice lacking lecithin:retinol acyltransferase (LRAT), the enzyme responsible for esterification of retinol, are frequently found to be infertile with hypoplasia of the testis and oligospermia. However, these LRAT knock-out mice had similar RA levels in their testes as wild type mice [42,43]. This might indicate a role for another retinoid besides RA in male fertility. Whether this effect is directly on the sperm cell or through an effect on for example Sertoli cells is not known.

1.3.3. Metabolic syndrome and NAFLD

Recently, interest emerges on the role of retinoids in the development of obesity and metabolic syndrome. Low levels of vitamin A were linked to an increase in adiposity [44–47]. RA has been shown to reduce adipogenesis and to promote lipid oxidation and insulin sensitivity. It has been proposed that RA exerts these effects by either RAR or peroxisome proliferator activated receptor (PPAR) β/δ, depending on the ratio between cellular RA binding protein 2 (CRABP2) and fatty acid binding protein (FABP5) (reviewed by [48]).

Non-alcoholic fatty liver disease (NAFLD), one of the symptoms of metabolic syndrome, is associated with reduced retinol serum levels and retinoic acid levels [49,50]. Normal retinoid signaling is important for preventing NAFLD, as was shown by interference with retinoid conversion or signaling: Transgenic mice that expressed a RA receptor (RAR) α– dominant negative form in hepatocytes have a reduced RAR/RXR heterodimer activity. These mice developed steatohepatitis and hepatocarcinogenesis. Feeding a RA rich diet prevented both the steatohepatitis and the liver tumors [51]. In another study deletion of one of the copies of retinol dehydrogenase 10 (Rdh10$^{+/-}$) in mice, causing a modest reduction of ATRA levels in liver and adipose tissue, led to a wide range of metabolic abnormalities. One of these abnormalities was an increase in liver steatosis after being fed a high fat diet. Also this phenotype could be rescued by feeding the mice ATRA [52].

It will be important to identify proteins through which retinoids are involved in the development of NAFLD. Human patient cohorts revealed several genetic variants associated with NAFLD, among which variations of patatin-like phospholipase domain-containing 3 (PNPLA3) and 17-β hydroxysteroid dehydrogenase 13 (HSD17B13) (reviewed by [53]). PNPLA3 has been reported to have retinyl-palmitate lipase activity, whereas HSD17B13 was shown to have retinol dehydrogenase activity [54,55].

Also retinol binding protein 4 (RBP4) has been linked to NAFLD. Overexpression of RBP4 in adipose tissue of mice led to an increased accumulation of fat in the liver after feeding a high fat diet and increased the amount of fatty acid uptake by the liver. It also increased inflammation in the adipose tissue [56]. On the other hand, suppression of RBP4 in liver and adipose tissue with RNA oligonucleotide against RBP4 reduced the amount of hepatic steatosis and triacylglycerol accumulation in mice on a high fat diet [57]. In several studies, associations between RBP4, adipocytes and insulin signaling, or obesity and diabetes have been reported [58–60], but how RBP4 contributes to these diseases and NAFLD remains unclear. Several factors make interpretation of the data difficult: 1) Some effects related to metabolic syndrome might be species specific and not translatable from rodents to humans; 2) Genetic differences within RBP4 have been reported [61]; 3) the distinction between apo and holoRBP4 might be relevant [62]; 4) RBP4 production within the liver or the adipose tissue and RBP4 in circulation *versus* local effects [63]; 5) Patients with diabetes often also exhibit renal insufficiency, which has been reported to influence RBP4 levels [64]; 6) RBP4 has recently been reported to bind fatty acids, which may be relevant for apoRBP4-mediated effects [65]. Nevertheless, several mechanisms for the connection between RBP4 and insulin resistance have been proposed. One mechanism is based on the observation that RBP4 overexpression in mice has been shown to induce insulin resistance through adipose tissue inflammation, whereas RBP4$^{-/-}$ mice are protected [66,67]. Another mechanism describes the binding of RBP4 to the Vitamin A receptor (STRA6), which has been shown to activate Janus kinase/signal transducers and activators of transcription (JAK/STAT) (reviewed by [68]). In summary, associations between retinoids, proteins and diseases have been reported, but mechanistically little is known. For a more detailed discussion we refer to a recent and extensive review by Blaner [49].

2. Uptake and entry into the circulation

2.1. Food sources

Mammals are not able to synthesize vitamin A and are dependent on supply through the diet. To meet vitamin A requirements, humans can eat animal-based food sources like liver, milk and butter. These sources contain retinyl esters, of which the liver, as the main storage site of vitamin A, contains the highest concentrations. Provitamin A, like β-carotene, can be a source of vitamin A from plant-based diets. Plants like carrot and spinach contain high amounts of β-carotene [69–71].

2.2. Uptake in the intestine and release into the circulation

The proximal small intestine is the main site of absorption of vitamin A. As vitamin A is a fat-soluble vitamin, absorption is facilitated by bile acids. Retinyl esters are hydrolyzed to retinol by retinyl ester hydrolases, which enzymatic activity is enhanced by bile acids (reviewed by [8]). Retinol is absorbed by enterocytes, either by diffusion or with the aid of a transporter (Fig. 2). A possible transporter involved might be retinol binding protein receptor 2 (RBPR2) [72]. Absorption of carotenoids was long thought to be a process of diffusion, but recently several proteins have been implicated in facilitating the uptake of carotenoids by enterocytes. The most likely candidates are Scavenger Receptor class B type I (SR-BI), Cluster Determinant 36 (CD36) and NPC1-like transporter 1 (NPC1L1) (Fig. 2). These protein candidates are also implicated in transporting other lipid molecules across the brush border into the enterocyte (reviewed by [70]).

After absorption, carotenoids can be converted to retinaldehyde by β-carotene-15,15′-monooxygenase (BCO1) [2] and subsequently to retinol by retinaldehyde reductases [73].

Free retinol in the enterocyte is bound to Cellular Retinol-Binding Protein 2 (CRBP2) [74] and can be esterified by LRAT or Diacylglycerol O-Acyltransferase 1 (DGAT1) into retinyl-esters [75] using different mechanisms. LRAT specifically uses the fatty acyl group at the SN-1 position of phosphatidylcholine (mostly palmitate), whereas DGAT1 uses acyl-CoA derived from the pool of free fatty acids, leading to differences in the fatty acid composition between retinyl esters produced by both enzymes [76–79]. Carotenoids and newly formed retinyl-esters within the enterocyte will be packed and released to the lymphatic

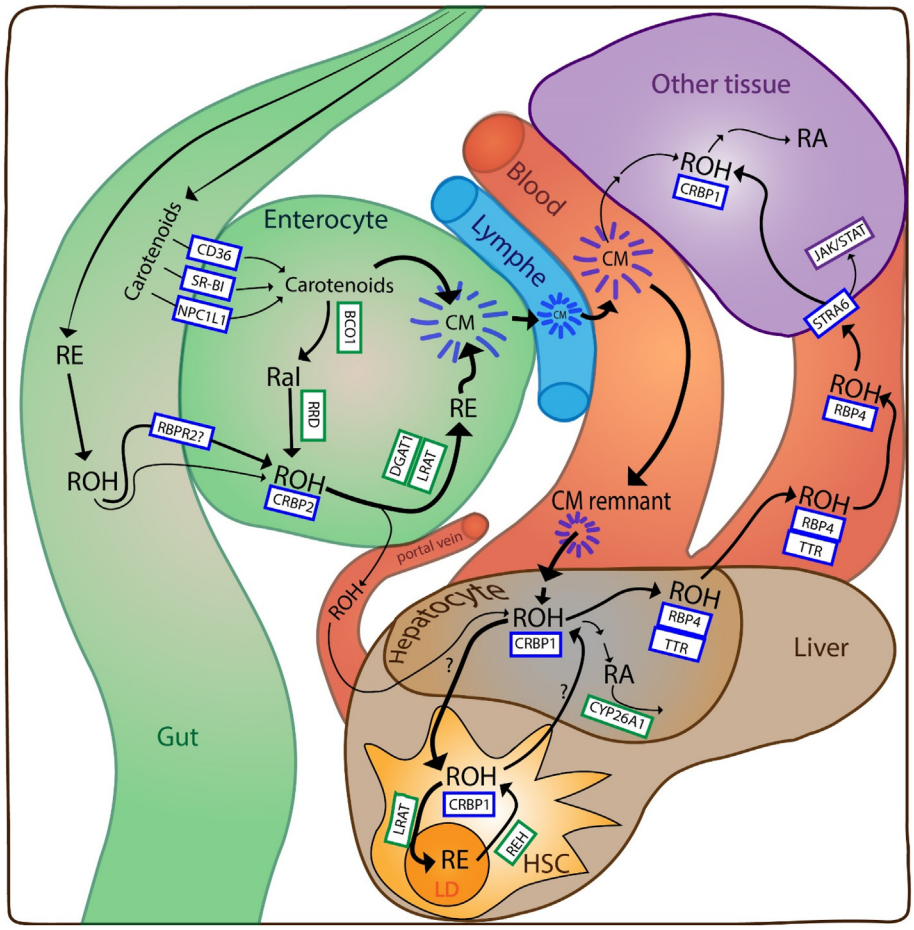

Fig. 2. Vitamin A metabolism: from food to liver and beyond. Metabolism of vitamin A after uptake from the diet as retinyl esters (RE) or carotenoids in the gut (green), *via* transport through the enterocyte, the lymph (blue) and blood (red), to the liver (brown), where it can be stored in hepatic stellate cells (HSC, yellow) or redistributed to other tissues (purple). For further explanation see text Sections 2 and 3. RE: retinyl esters, ROH: retinol, Ral: retinaldehyde, RA: retinoic acid, CD36: Cluster Determinant 36, SR-BI: Scavenger Receptor class B type I, NPLC1L1: NPC1-like transporter 1, RBPR2: retinol binding protein receptor 2, BCO1: β-carotene-15,15′-monooxygenase, RRD: retinal reductase, CRBP2: Cellular Retinol-Binding Protein 2, DGAT1: Diacylglycerol O-Acyltransferase 1, LRAT: lecithin:retinol acyltransferase, CM: chylomicron, CRBP1: Cellular Retinol-Binding Protein 1, RBP4: retinol binding protein 4, TTR: transthyretin, CYP26A1: Cytochrome P450 Family 26 Subfamily A Member 1, STRA6: receptor stimulated by retinoic acid 6, JAK/STAT: Janus kinase/signal transducers and activators of transcription, LD: lipid droplet, HSC: hepatic stellate cell, REH: retinyl hydrolases (generic term, for several specific enzymes see text).

system in chylomicrons (CM), and through the lymphatic system they will enter the bloodstream (reviewed by [8,70]) (Fig. 2). The retinoids can be taken up by tissues, for example by the lung, spleen and adrenal glands, or they can be transported to the liver incorporated in chylomicron remnants [80] (Fig. 2). Some reports suggest that retinol absorbed in the intestine by diffusion is not excreted by CMs but directly released in the portal circulation. However, the evidence for this suggestion mostly comes from early studies or studies in cell culture [81–84].

3. Liver storage and release to maintain vitamin A homeostasis

The CM remnants still contain large amounts of retinyl-esters and are taken up by hepatocytes [85] (Fig. 2). The retinyl-esters are thought to be hydrolyzed in late endosomes with concomitant release of free retinol in the cytosol [86]. Free retinol in hepatocytes can have several destinations: i) release into circulation (Section 3.1); ii) conversion to retinoids (Section 3.2); and iii) transfer to HSCs (Section 3.3). After transport to HSCs, retinol is stored as retinyl ester in large lipid droplets (LDs), contributing to the buffering capacity of the liver (Section 3.4).

3.1. Release into circulation

Retinol can be found in the cytosolic fraction where it can be bound by CRBP1 [87,88]. Possibly this is the mode of transport towards the endoplasmic reticulum (ER), where retinol binds to RBP4 and transthyretin (TTR) [89,90]. From the ER the complex is transported through the Golgi into secretory vesicles and released into the circulation. The transfer of RBP4 from the ER to the Golgi is dependent on the presence of retinol [89–91]. Hepatocytes are the main source of RBP4 in the circulation of mice [63]. When there is no TTR present, RBP4 levels in the liver increase with 60%, suggesting a role for TTR in the release of RBP4 from the hepatocyte [92]. TTR is stabilized by forming a complex with RBP4 and retinol (holoRBP), although it can also bind RBP4 without retinol (apoRBP) with a lower affinity. TTR has also been shown to bind RA without RBP4 [93]. Traditionally, TTR was seen as a protein that prevented loss of RBP4 from the circulation by filtration in the kidney. Recently, TTR was also shown to influence the binding of holoRBP4 to the receptor stimulated by retinoic acid 6 (STRA6) [94]. STRA6 is a membrane receptor that binds RBP4 to facilitate transport of retinol across the membrane [95]. Data from STRA6 knock-out mice indicate that the eye, brain and testis depend on retinol transported *via* STRA6 [96]. The eye is completely unable to take up retinol without STRA6. Since STRA6 requires retinol to be bound by RBP4 in order to transport it into the cell, this makes the eye very dependent on retinol processing by the liver, where retinol is exported together with RBP4. Under normal feeding conditions other tissues can, at least partially, take up retinyl esters from CMs. When put on a vitamin A-deprived diet, the testis and the brain also require STRA6 to maintain retinol levels. But in these two organs the STRA6 expression is regulated by retinoic acid amounts, reducing the STRA6 expression during vitamin A-deprivation, redirecting the RBP4-retinol towards the eye [96]. STRA6 is not only translocating retinol from holoRBP towards apoCRBP1 in the cell, activation of STRA6 in this process also activates JAK/STAT signaling (Fig. 2) [97]. One of the effects of this signaling function might be a reduction of the responsiveness to insulin through affecting the suppressor of cytokine signaling 3 (SOCS3) [98]. From the combined recent reports on STRA6, together with the older studies on diffusional uptake of retinol, emerges the idea that the main route of retinol uptake into cells is trough diffusion [99] and that STRA6 plays a

role in retinol transport in tissues with a high demand, like the retina (but not the liver [94]) and in situations where the holoRBP/TTR ratio concentration is increased. This would leave holoRBP free to interact with STRA6 without inhibition by TTR. Besides from transporting retinol, in these situations STRA6 also activates signaling cascades.

Another set of proteins able to transport retinol are serum amyloid A (SAA) proteins. These proteins are expressed in the liver and intestine, were the levels of expression are correlated to the amount of retinol. They increase in amount during (bacterial) infection and the free form (not bound to high density lipoproteins) is able to transport retinol through the circulation, allowing retinol to systemically exert its functions in the immune response [100,101]. The expression of SAA in the intestine is controlled by RARβ [102].

3.2. Conversion to retinoids

Hepatocytes, like many other cell types, are able to produce RA. To produce RA retinol first needs to be converted to retinaldehyde by retinol dehydrogenases (RDH). Many enzymes have been reported to possess RDH activity, although probably not all of them are physiologically relevant (reviewed by [103]). Recently HSD17B13 has also been shown to have RDH activity and to be expressed in hepatocytes [54]. Hepatocytes express four versions of retinaldehyde dehydrogenase (RALDH), although not all hepatocytes express all RALDHs. These enzymes can produce RA from retinaldehyde [104]. The conversion of retinol to RA can also be used to prevent vitamin A toxicity in response to increased dietary uptake. Indication for this comes from studies using alcohol dehydrogenase (ADH) knock-out mice, with ADH1 and ADH3 knock-out mice showing increased vitamin A toxicity in response to dietary supplementation, with lower RA levels in the liver [105–108]. Active RAs can be broken down by CYP26A1 into inactive RA metabolites. The expression of this enzyme is also regulated by RA and its metabolites, which in this way regulates itself [109,110].

3.3. Transfer to HSCs

Around 80% of the vitamin A present in the body is stored as retinyl esters in LDs within HSCs (reviewed by [111]). In times of a shortage of retinol supply through the diet, these retinyl ester stores in the HSC can be utilized. The mechanisms behind retinol transfer between hepatocytes and HSCs are not clear yet (Fig. 2) [111]. For a long time RBP4 was implicated in this transport. Blomhoff et al. reported in 1988 that by blocking RBP4 receptors in the HSC with antibodies, they could prevent transfer of retinol from hepatocytes to HSCs [112]. But RBP4$^{-/-}$ mice store similar or even increased amounts of retinyl esters in the liver, although retinol levels in serum are below the detection limit [113,114]. This indicates that RBP4 is not required for transport of retinol from the hepatocyte towards the HSC, but that it is indispensable for release and transport of retinol from the liver towards other tissues. Remarkably, RBP4 must be synthesized within the liver to be able to transport liver-stored retinol as extrahepatically expressed RBP4 cannot substitute for endogenously expressed RBP4 in the liver [114]. Reports on the question whether HSCs can produce and excrete RBP4 themselves, are conflicting [115,116]. STRA6 expression was not detected in liver homogenates, but recently a new RBP4 receptor has been discovered called retinol binding protein receptor 2 (RBPR2). This receptor is mainly expressed in liver and intestine, and knockdown of RBPR2 in cultured hepatocytes causes a clear decrease in the uptake of retinol bound to RBP4. In the livers of mice the expression of RBPR2 is correlated to the amount of retinyl-esters [72]. Another hypothesis is that retinol is transferred between hepatocytes and HSCs by direct cellular contact through gap junctions, possibly by CRBP1 [111,117].

3.4. Retinol in hepatic stellate cells; LD dynamics and the role of LRAT

In HSCs retinol is transported to LRAT by CRBP1 for esterification and storage in LDs [117]. LDs within the HSC are specialized for storing excess dietary retinol as retinyl-esters. For instance, in rats on a regular diet, HSC LDs contain 39.5% retinyl ester; 31.7% triglyceride; 15.4% cholesteryl ester; 4.7% cholesterol; 6.3% phospholipid; and 2.4% free fatty acids [118]. The amount of retinyl-esters is strongly influenced by the uptake from the diet, whereas the amounts of TAG in HSC LDs are not responsive to changes in TAG uptake from the diet [4]. LRAT is the enzyme mainly responsible for the formation of retinyl-esters from retinol. In the absence of LRAT another enzyme, DGAT1, is also able to produce retinyl-esters, although at much lower efficiency [42,79,119]. LRAT seems to be essential for creating the large LDs, which are characteristic for quiescent HSCs. DGAT1 might be involved in creating small LDs, which contain less retinyl esters and which have a more rapid turnover in comparison with to the large LDs [119,124]. Recent studies show that retinyl ester synthesis by LRAT is sufficient to drive the formation of retinyl ester filled LDs [120]. The transmembrane domain in the C-terminus of LRAT is embedded in the endoplasmic reticulum, directing newly formed retinyl esters into the lipid bilayer of the ER. The N-terminus of LRAT is essential for the formation of the large LDs and has affinity for retinyl esters [120]. The level of gene expression of LRAT is responsive to the levels of retinol present outside the HSC and increasing these retinol levels increases LRAT activity [121]. To release retinol from the stores inside the LDs of the HSC, several hydrolases have been implicated, including adipose triglyceride lipase (ATGL), patatin-like phospholipase domain-containing 3 (PNPLA3) and hormone sensitive lipase (HSL), but a definitive answer on how retinyl esters are hydrolyzed has not been found yet [55,122,123]. Another mechanism that might be involved in breakdown of the large LDs and the release of retinol could be through autophagy [124–127].

4. Vitamin A in liver pathology

4.1. Release of retinol and retinoid acid during acute liver damage

Multiple liver diseases have been associated with HSC activation, among which NAFLD, alcohol induced liver disease and viral infections like hepatitis B and hepatitis C (reviewed by [128]). Activation of HSCs can occur through multiple pathways (for a quick overview [129]). During the activation process, HSCs lose their large LDs to become myofibroblast-like cells that secrete extracellular matrix proteins like collagen (Fig. 3). The concomitant loss of RE storage during HSC activation is well documented, both *in vitro* during cell culture on plastic [130–132] and *in vivo* using rodent models with carbon tetra chloride (CCL_4), thioacetamide (TAA) or partial hepatectomy (PHE), measuring RE in total liver [119,133,134]. During activation of HSCs, retinyl ester levels within the cell decrease together with the TAG levels. However, the relative amount of polyunsaturated fatty acid (PUFA) containing TAG species increases [130,135]. This happens under the influence of acyl CoA synthetase (ACSL) type 4, which converts free, preferentially long-chain and unsaturated fatty acids to acyl-CoAs [136]. These TAGs are present in small LDs that appear during the activation process and have a high turnover, with DGAT1 and ATGL having a role in buildup and breakdown, respectively [137]. The breakdown of the large LDs is thought to happen at lysosomes by lysosomal acid lipase [127]. The released retinol and fatty acids can partially be re-used to create new small LDs [124,127] (Fig. 3).

Part of the released retinol is thought to be dehydrogenated towards retinaldehyde and subsequently converted to retinoic acid. In rats treated with the liver-toxins CCL_4 or TAA, retinoic acid amounts within complete liver have been shown to be increased [133,138]. Mice lacking RE stores because of LRAT deficiency showed a delay in the initial regeneration of the liver after PHE. Although in wild type mice the RA levels did not increase in response to PHE, the RA levels in the livers of the LRAT$^{-/-}$ mice were lower, as well as the expression of retinoid-responsive genes. Hence, the authors concluded that the delay

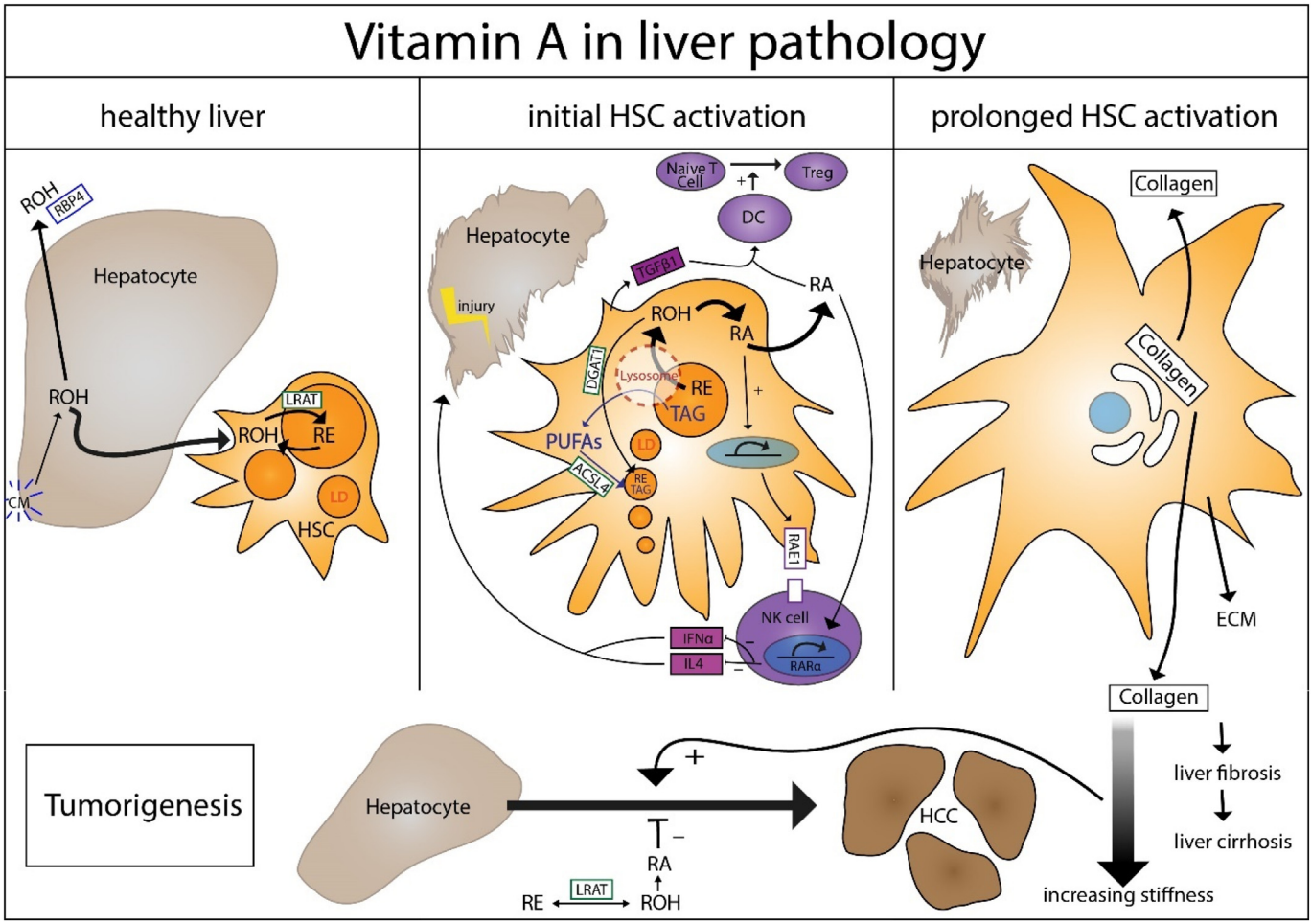

Fig. 3. Retinoids in liver sickness and in health. Retinoid storage and release in a healthy liver (left), retinoid release during initial HSC activation in response to liver damage (middle), role of HSCs after prolonged activation (right), and the influence of HSC activation and retinoids on tumorigenesis (bottom). For further explanation, see Section 4. RE: retinyl esters, ROH: retinol, RA: retinoic acid, CM: chylomicron, LD: lipid droplets, RBP4: retinol binding protein 4, LRAT: lecithin:retinol acyltransferase, HSC: hepatic stellate cell, DC: dendritic cell, DGAT1; Diacylglycerol O-Acyltransferase 1, PUFAs: poly-unsaturated fatty acids, ASCL4: acyl CoA synthetase type 4, TAG: triacylglycerol, NK cell: natural killer cell, RAE1: retinoic acid early inducible gene 1, RARα: Retinoic acid receptor α, IFNα: interferon α, IL4: interleukine 4, ECM: extracellular matrix HCC: hepatocellular carcinoma.

in liver regeneration after PHE was caused by the lack of RE stores available for RA production [134]. In this study, it was assumed that hepatocytes are responsible for RA production, but recently a model was proposed where next to hepatocytes also Kupffer cells, cholangiocytes, liver sinusoidal endothelial cells and HSCs produce RA [139]. It remains to be established whether all these cell types can produce RA, but HSCs do seem to be able to produce RA. First of all, both mice and human HSCs express ADH1 [140,141] and mice HSCs have been shown to increase the expression of alcohol dehydrogenase 3 (ADH3) during activation in culture [142,143]. ADH1 and ADH3 are able to oxidize retinol into retinaldehyde [144]. Subsequently, retinaldehyde must be oxidized to RA and HSCs from mice express RALDH1 and RALDH2, with RALDH2 gene expression increasing in the first 7 days of culture activation [131,142]. Secondly, several studies measure RA content of HSCs [131,138,143,145,146] and an increase in RA production during activation was observed in several cases [131,138,143]. One of these studies combined intracellular measurements of retinol and RA in mouse HSC cell cultures at four different timepoints with gene expression levels of the activation markers RALDH1, RALDH2 and RAR and RXR isoforms. It was found that RA production peaks at day 4, matching the detection of ATRA in the medium. After prolonged activation, retinyl-ester stores were lost and RA levels decreased again [131]. Thus, part of the retinyl ester stores in HSCs is used to produce RA during initial HSC activation and HSCs are able to contribute to RA production.

What could be the function of increased RA levels during initial HSC activation? Below we will briefly discuss an effect of retinoids on liver cells and on immune cells.

4.1.1. RA to increase hepatocyte proliferation and decreasing HSC proliferation and activation

RXRα deficiency in hepatocytes and RA administration through the diet have opposite effects, fitting with the notion that RA exerts its effects through RXRα. RA induces the synthesis of unsaturated fatty acids and phospholipids and breakdown of TAG [147]. This increase in unsaturated fatty acid and the breakdown of TAG in total liver is exactly what occurs in activating HSCs during the initial stage of activation *in vitro* [130,135,136]. It has also been shown that RA can reduce collagen and TGFβ production in HSCs and that it has an anti-proliferative effect on HSCs [148–151]. RA also reduces the amount of liver fibrosis in mice after treatment with CCL_4 and increases survival [149]. On the other hand, RA increases proliferation of hepatocytes, both in healthy liver and after induction of liver regeneration [152–154]. Thus, it seems that upon liver injury, HSCs release their retinyl ester stores to produce an increase in RA, which amplifies the regeneration of the liver by increasing hepatocyte proliferation and decreasing HSC proliferation and activation. In support of this idea it was found that $LRAT^{-/-}$ mice, which lack the large retinyl ester stores,

show a delay in regeneration after hepatectomy [134].

4.1.2. RA to modulate the immune response

Another role of RA released by the activating HSCs might be to affect the immune system [155]. Some studies looked at the interaction between HSCs and immune cells (DCs and T cells) and reported a role for RA in inducing Foxp3$^+$ regulatory T cells in the presence of DCs and TGFβ1 [141,156]. Although RA was not measured directly in these studies, the effect on T cells was lost by adding a RAR antagonist or by using HSCs from vitamin A deficient mice, whereas the effect can also be mimicked by adding RA in the absence of HSCs. However, rather than RA production by HSC directly, it cannot be excluded that retinol released from the HSCs is converted to RA by other cell types in the co-culture experiments. RA can also induce expression of retinoic acid early inducible gene 1 (RAE1) in HSCs. RAE1 is an activating ligand for natural killer cells and its expression leads to an increased susceptibility of early stage activating HSCs to be killed by NK cells [131]. RA, through RAR-α, can decrease the production of IFN-γ and IL-4, but not TNF-α, by NK cells. The reduction of these cytokines can reduce hepatocyte cell death after experimental induction of hepatitis with Con A, but not after induction with α-GalCer [24]. These effects also point to a role for RA released by HSCs in the early stages of HSC activation in limiting the amount of fibrosis formation and the immune response, favoring hepatocyte proliferation and survival as well as liver regeneration (Fig. 3).

4.2. Prolonged liver insult, prolonged HSC activation, and tumorigenesis

When the cause of the liver damage is not resolved, excessive amounts of collagen production can lead to the development of liver fibrosis, with liver cirrhosis as its end stage. Patients with chronic liver disease like hepatitis C have lower serum retinol levels than healthy subjects [157]. Under chronic conditions, HSCs have switched from an initial to a prolonged/chronic activation state [158,159]. Prolonged activated HSCs have long lost their retinyl ester stores and lose their susceptibility to NK cell killing, mediated by the loss of RAE1 [131]. Prolonged activated HSC become unresponsive to retinoids, as measured by their αSMA, RAR α, RARβ and RARγ expression responses upon incubation with retinol or RA [132]. The production of extracellular matrix (ECM) components by these HSCs can lead to a positive feedback loop, maintaining HSC activation and inducing more ECM production (reviewed by [160]).

A cirrhotic liver becomes stiff, and liver stiffness is associated with an increased risk for development of hepatocellular carcinoma (HCC) [161]. Cells can sense stiffness of their surroundings, for example by proteins like integrins. Within HCC cell lines, matrix stiffness has been shown to promote proliferation, to increase chemotherapeutic resistance [162] and to influence the activation of TGFβ1 through the β1 integrin-FAK-Rho GTPase pathway [163]. TGFβ1 is associated with tumor progression of HCC [164]. HCC is the most common cause of death in patients that have developed liver cirrhosis. HSCs also influence the development, invasion and angiogenesis of HCC by their ECM production and other factors. They are thought to be the origin of cancer associated fibroblasts (reviewed by [165,166]).

There seems to be a relation between retinoids and HCC. In human cohort studies, the levels of retinol and retinaldehyde in tissue and serum decreased in the order of normal to cirrhosis to HCC, allowing to differentiate between HCC and cirrhosis in tissue samples and in serum [167]. In addition, RA levels were lower in HCC tissue and serum as compared to healthy and cirrhotic samples. In contrast to the stimulatory effect of RA on hepatocyte proliferation, several studies show that RA causes a reduction of tumor formation, metastasis and proliferation and an increase in differentiation of tumor cells [168–171]. In mice where the RARα has been disrupted specifically in hepatocytes, livers show hepatosteatosis and the mice have a high incidence of HCC. When these mice were fed a RA rich diet, they showed an almost normal incidence of these pathologies [51]. Treatment of HepG2 cells with sorafenib (a drug used against HCC) in combination with ATRA induced more cell death as compared to treatment with sorafenib alone. ATRA induces AMPK activation and reduces intracellular ATP levels, suggesting that apoptosis was induced by activation of the AMPK–p38 MAPK pathway [172]. This all points to RA inhibiting the formation and the growth and survival of HCC. Other evidence for a role for retinoids in HCC comes from mice that are lacking LRAT and therefore lack almost all RE storage in the liver. In these mice hepatic tumorigenesis was reduced [173,174]. At first sight this seems contradicting with the protective effects of RA, but the expression of enzymes involved in retinoid signaling in these mice in response to tumor induction was increased. The expression of CYP26A1 and RARβ, both known to be regulated by retinoid amounts, as well as the expression of cyclin-dependent kinase inhibitor p21, which has an antiproliferative effect and is responsive to RA was increased in LRAT$^{-/-}$ mice [173]. This could indicate that LRAT$^{-/-}$ mice have higher RA levels as compared to wild type mice. In agreement with this, similar observations were made with CRBP1$^{-/-}$ mice in which transport of retinol to LRAT is impaired, reducing storage of retinol as retinyl esters [117]. After injection of retinol, CRBP1$^{-/-}$ mice have higher RA and lower RE liver levels compared to wild type mice. These effects were opposite from injection of retinol in ADH1$^{-/-}$ mice, which had lower RA and higher RE liver levels [106]. This suggests that within the liver, CRBP1 and ADH1 exhibit opposite roles and convert free retinol to either RE or RA. These mechanisms may be relevant during conditions with reduced or excess vitamin A levels [106,108]. A similar correlation between LRAT, retinoic acid, and tumor formation as in LRAT$^{-/-}$ mice was observed in melanoma cells. Within these melanoma cells, LRAT expression is also upregulated, protecting them from the antiproliferative effects of ATRA. In addition, overexpression of LRAT reduced the intracellular levels of ATRA [175].

From the above-mentioned studies the concept arises that retinyl ester storage within HSCs might not only be a buffer to deal with shortage of retinol availability from the diet, but that it also buffers production levels of RA. Increases in the amount of RA lead to an increased expression of CYP26A1 for breakdown of RA [110] as well as to increased expression of LRAT for esterification of retinol, thereby preventing more retinol to be converted to RA [176,177]. When LRAT is not present anymore (*e.g.* after activation of HSCs), the RE buffer is lost and (local) increased concentrations of RA could be the result. Subsequently, increased RA levels can inhibit HCC formation through its antiproliferating and pro-differentiation effects [168–171].

5. Conclusions

Vitamin A is involved in many different biological processes. It is important for growth, vision and reproduction. It is involved in the immune system and we are only starting to understand its role in metabolic syndrome (obesity, diabetes, NAFLD). Besides the precise roles of the known active retinoids like RA, the signaling functions of ROH bound to RBP4 deserve attention in future research. How retinol is transported between hepatocytes and HSCs and back again remains a mystery. Possibly, a specific transport protein is involved, or the transport is mediated by the ROH-binding protein CRBP that shuttles *via* tight junctions between hepatocytes and HSCs.

In case of liver damage, HSCs release large amounts of the stored vitamin A, at least partially as RA. The effects of this RA surge both on the HSC itself and on the neighboring cells within the liver have been studied to a limited extend. The surge of RA can mediate the immune response by influencing NK cells, and it can increase the regenerative capacity of the liver. The mechanisms by which RA influences DCs and T cells are still largely unknown, and it is also not clear how these immune cells in turn affect hepatocytes. It has also been observed that although RA causes a decrease in the severity of liver injury, this depends on the type of liver injury sustained [24]. This illustrates yet

another challenge, since most studies are carried out on models using only one type of liver injury. Hence, it is possible that mechanisms unraveled within one model can only be applied to a subset of potential causes of liver damage.

If liver damage persists, activated HSCs gradually change into myofibroblast-like cells, producing extracellular matrix. The liver becomes stiffer, which increases the chances of tumorigenesis to occur. There is an increasing amount of evidence regarding the interaction of HCC and HSCs. The HSC-induced substrate stiffness and retinoid-release are clearly involved in tumorigenesis, but the poorly defined different states of HSCs are a complicating aspect. RA has been shown to decrease proliferation and metastasis of hepatocellular carcinoma. The levels of RA and RA signaling are influenced by the possibility to esterify retinol towards RE. This suggests a complex regulation between different types of retinoids, with an important role for RE storage in HSCs. The conversion between the different types of retinoids seems to be critical in many systems, emphasizing the need for research in systems able to integrate the physiological complexity.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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