Review

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Augmenter of liver regeneration: Mitochondrial function and steatohepatitis

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Summary

Augmenter of liver regeneration (ALR), a ubiquitous fundamental life protein, is expressed more abundantly in the liver than other organs. Expression of ALR is highest in hepatocytes, which also constitutively secrete it. *ALR* gene transcription is regulated by NRF2, FOXA2, SP1, HNF4α, EGR-1 and AP1/AP4. ALR's FAD-linked sulfhydryl oxidase activity is essential for protein folding in the mitochondrial intermembrane space. ALR's functions also include cytochrome c reductase and protein Fe/S maturation activities. ALR depletion from hepatocytes leads to increased oxidative stress, impaired ATP synthesis and apoptosis/necrosis. Loss of ALR's functions due to homozygous mutation causes severe mitochondrial defects and congenital progressive multiorgan failure, suggesting that individuals with one functional *ALR* allele might be susceptible to disorders involving compromised mitochondrial function. Genetic ablation of *ALR* from hepatocytes induces structural and functional mitochondrial abnormalities, dysregulation of lipid homeostasis and development of steatohepatitis. High-fat diet-fed ALR-deficient mice develop non-alcoholic steatohepatitis (NASH) and fibrosis, while hepatic and serum levels of ALR are lower than normal in human NASH and NASH-cirrhosis. Thus, ALR deficiency may be a critical predisposing factor in the pathogenesis and progression of NASH.

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Key point

ALR is an evolutionally conserved protein that is abundantly expressed in hepatocytes.

Introduction

The liver has a remarkable ability to restore its mass after major partial resection.[1] During attempts to discover growth factors responsible for this phenomenon, **a**ugmenter of **l**iver **r**egeneration (ALR) was identified in the extracts of regenerating livers.[2,3] It was postulated that hepatocyte-stimulatory or regeneration-augmenting activity was present in the hyperplastic and regenerating livers.[2,4] However, ALR's function in physiology was suggested by subsequent research that observed equivalent expression of the protein in the hepatocytes of weanling and resting adult livers.[5] Expression of ALR in non-parenchymal hepatic stellate cells, Kupffer cells and endothelial cells is much lower than in hepatocytes.[5] Cholangiocytes also express ALR.[6] Although most of the ALR-related research has been limited to its function in the liver, the presence of ALR in the heart, brain, lung, kidney, skeletal muscle, spleen and testes[7–9] suggests that it has important roles in the physiology and pathophysiology of other organs as well.

Hepatocytes produce and secrete ALR constitutively,[5,10] and intracellular ALR is found in the mitochondria, nucleus and cytosol.[11,12] A critical role of ALR in mitochondria is evident as its loss *in vitro* and *in vivo* causes lipid accumulation (steatosis), ATP depletion, oxidative stress, mitochondrial degeneration, and death of hepatocytes.[13–16] ALR also inhibits mitochondrial membrane permeability transition, thus protecting cells from injury.[17] Interestingly, hepatic steatosis of various aetiologies is associated with down-regulation of ALR expression.[14–16,18,19]

Non-alcoholic fatty liver disease (NAFLD) has become a major clinical challenge of recent times. NAFLD begins with simple steatosis that can progress to the more aggressive form, non-alcoholic steatohepatitis (NASH) in 10-30% of affected individuals.[20] A significant number of patients with NASH develop cirrhosis and some may progress to hepatocellular carcinoma (HCC) with or without cirrhosis.[21,22] Since disordered mitochondrial function is a critical component of NAFLD pathophysiology,[23–26] it is apparent that ALR deficiency may play an important role in its pathogenesis and progression. In this review, we discuss the current understanding of the regulation of ALR expression and function, and we review the possible mechanisms by which ALR deficiency might contribute to aggressive NAFLD.

Augmenter of liver regeneration gene and isoforms

Although known as augmenter of liver regeneration, hepatopoietin and hepatic stimulatory substance, these names are rather misnomers since ALR is present ubiquitously (in all major organs) and demonstrates functions other than liver cell

proliferation.[9,11,12,27] However, the highest expression of ALR is found in the liver and testes.[7–9,28] The *ALR* gene is mapped to chromosome 16 (human), 17 (mouse) or 10 (rat).[7,8,28] The highly conserved ALR gene and protein sequences in humans, mice and rats, and the presence of homologous proteins in yeast,[28] insects,[29] and viruses[30] indicate that the gene is evolutionally conserved. Homology between the rat, mouse and human ALR and the yeast scERV1 protein is shown in Fig. 1.

The mammalian *ALR* gene is named *GFER* (growth factor Erv1-like) because of its structural and functional similarities with the scERV1 (**e**ssential for **r**espiration and **v**egetative growth-1) protein expressed by the yeast *Saccharomyces cerevisiae*.[7,8,28,31] The human *ALR* is a single copy gene mapped to chromosome 16 in the polycystic kidney disease locus.[28] Two major forms of ALR, short (~15 kDa; s-ALR) and long (~22 kDa; l-ALR), have been identified.[5,16,19,32,33] However, the presence of multiple ATG codons in the *ALR* gene suggests the possibility of variants generated by alternative splicing. The entire mouse *ALR* gene is contained in a 6.7-kb *Hind*III fragment, comprising 3 exons and 2 introns.[8] Exon 1 contains a 5' untranslated sequence, ATG initiation codon and 6 amino acid-coding nucleotide sequence of s-ALR or 73 amino acid-coding sequence of l-ALR, which is followed by a 400 bp intron; exon 2 contains a 66 amino acid-coding sequence, followed by the second 480 bp intron; the third exon contains the remaining portion of the amino acid-coding sequence and the entire 3' untranslated sequence.[8] The N-terminal domain of l-ALR contains the mitochondrial leader sequence (absent in s-ALR), whereas the C-terminal domain is responsible for the flavin adenine dinucleotide (FAD)-linked functional activity.

ALR gene expression regulation

The *ALR* gene has a TATA-less promotor with features of housekeeping genes, oncogenes, growth factors and transcription factors.[34] Analysis of the *ALR* promoter identified positive regulatory elements between -416 and -608 nucleotide (nt), negative regulatory elements between -236 and -416 nt, and minimal core promoter activity between −22 and +27 nt. A "CTGGAGGC" sequence within the initiator (Inr)-like element and 2 other tandem flanking repeats comprise the core promoter that controls transcriptional initiation and the constitutive expression of the *ALR* gene. Activator protein 1/4 (AP1/AP4) is proposed to be responsible for basal *ALR* promoter activity.[35] *ALR* gene expression is positively regulated by nuclear factor erythroid 2-related factor 2 (NRF2), forkhead box A2 (FOXA2 also known as hepatocyte nuclear factor 3β [HNF3β]), HNF4α, early growth response protein-1 (EGR-1) and specificity protein 1 (SP1). An antioxidant response element (ARE) is located at −27/−19 nt from the initial ATG codon in the

A

Accession	Description	Amino acid length	Sequence similarity
P56213	FAD-linked sulfhydryl oxidase ALR [*Mus musculus*]	198	100
Q63042	FAD-linked sulfhydryl oxidase ALR [*Rattus norvegicus*]	198	95.45
P55789	FAD-linked sulfhydryl oxidase ALR [*Homo sapiens*]	205	74.76
P27882	Mitochondrial FAD-linked sulfhydryl oxidase ERV1; AltName: Full=Essential for respiration and vegetative growth protein 1 [*Saccharomyces cerevisiae* S288C]	189	47.71

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mALR    1   MAAPSEPAGFP-----RGSRFSFLPGGARSEMTDDL-----VTDARGRGARHRDDTTPAAAPAPQGLEHG--------KR   62
rALR    1   MAAPSEPAGFP-----RGSRFSFLPGGAHSEMTDDL-----VTDARGRGARHRKDNAPAAAPAPKGLEHG--------KR   62
hALR    1   MAAPGERGRFH-----GGNLF-FLPGGARSEMMDDL-----ATDARGRGAGRRDAAASASTPAQAPTSDSPVAEDASRRR   69
scERV1  1   MKAIDKMTDNPPQEGLSGRKIIYDEDGKPCRSCNTLLDFQYVTGKISNGLKNLSSNGKLAGTGALTGEAS----------   70

mALR    63  PCRACVDFKSWMRTQQKRDIKFREDCPQDREELGRHTWAFLHTLAAYYPDRPTPEQQQDMAQFIHIFSKFYPCEECAEDI  142
rALR    63  PCRACVDFKSWMRTQQKRDIKFREDCPQDREELGRNTWAFLHTLAAYYPDMPTPEQQQDMAQFIHIFSKFYPCEECAEDI  142
hALR    70  PCRACVDFKTWMRTQQKRDTKFREDCPPDREELGRHSWAVLHTLAAYYPDLPTPEQQQDMAQFIHLFSKFYPCEECAEDL  149
scERV1  71  ------ELMPGSRTYRKVD-------PPDVEQLGRSSWTLLHSVAASYPAQPTDQQKGEMKQFLNIFSHIYPCNWCAKDF  137

mALR    143 RKRIGRNQPDTSTRVSFSQWLCRLHNEVNRKLGKPDFDCSRVDERWRDGWKDGSCD  198
rALR    143 RKRIDRSQPDTSTRVSFSQWLCRLHNEVNRKLGKPDFDCSRVDERWRDGWKDGSCD  198
hALR    150 RKRLCRNHPDTRTRACFTQWLCHLHNEVNRKLGKPDFDCSKVDERWRDGWKDGSCD  205
scERV1  138 EKYIRENAPQVESREELGRWMCEAHNKVNKKLRKPKFDCNFWEKRWKDGWDE----  189
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Fig. 1. Sequence homology between mouse, rat, human ALR and Saccharomyces cerevisiae scERV1 protein. Sequence alignment was performed using Blast 2 sequences program (https://blast.ncbi.nlm.nih.gov/). (A) Amino acid length, and sequence similarity in ALR gene among mouse, rat, human and yeast. (B) Amino acid sequence alignment between rat, mouse and human ALR, and scERV1.

proximal promoter region. Upregulation of ALR expression by oxidative stress (due to an increase in nuclear NRF2 and its binding to ARE) suggests that *ALR* is an ARE-regulated gene.[36] FOXA2 binds at +276/+282 nt in the intronic promoter and its binding is amplified by the IL-6 response element at +265/+271 nt.[37] SP1 binds at −152/−145 nt and its overexpression markedly elevates ALR expression.[38] The *ALR* promoter also contains 2 potential bile acid-binding response elements, and bile acids suppress *ALR* promoter activity induced by FOXA2, HNF4α (binding site at +421/+432 nt) and EGR-1c (binding site at +304/+314 nt) via activation of small heterodimer partner (SHP).[39]

Key point

ALR loss causes oxidative stress, ATP depletion, mitochondrial degeneration and death of hepatocytes.

Interestingly, binding of HNF4α at -209/-204 nt negatively regulates *ALR* promoter activity.[38] CCAAT/enhancer binding protein-β (C/EBPβ) is another negative regulator of *ALR* gene transcription. In HepG2 cells, electrophoretic mobility-shift assay and chromatin immunoprecipitation analysis revealed a C/EBPβ-binding site at −292/−279 nt, and the epidermal growth factor (EGF) was found to downregulate ALR expression via C/EBPβ.[40] It is apparent that altered ALR expression due to variable activation of these transcription factors may influence pathophysiological changes during disease progression.

ALR's role in cell viability and growth

Hepatic ALR is transiently decreased following 70% (but not 40%) hepatectomy in normal rats, with a corresponding increase in its serum concentration.[5] ALR administration augments liver regeneration after 40% but not 70% hepatectomy.[4,41] This suggested that ALR released after 70% hepatectomy stimulates synthesis of growth mediators. Thus, the augmenting effect of exogenous ALR is proposed to be due to ALR-induced synthesis of tumour necrosis factor (TNF)α and IL-6 by Kupffer cells,[41] which prime hepatocyte regeneration.[42] Furthermore, ALR reduced oxidative stress, autophagy and apoptosis, and at the same time increased oxidative phosphorylation and mitochondrial expression of ATPase 6/8, ND1 subunit and mitochondrial transcription factor A (TFAM) in partially hepatectomised rats.[33,43]

Key point

ALR is essential for proper folding of mitochondrial imported proteins, electron transport chain activity and iron/sulphur maturation of cytosolic proteins.

Intracellular ALR was found to be essential for the survival of murine hepatocytes[13] and human hepatoma HepG2 cells.[44] Increasing ALR expression either through activation of transcription factors (*e.g.*, NRF2) or via plasmid transfection was found to be pro-proliferative and anti-apoptotic.[36] The *in vivo* relevance of these findings is demonstrated by robust apoptosis of hepatocytes upon genetic ablation of ALR expression.[14]

Early studies showed that partially purified as well as cloned ALR protects the liver from portacaval shunt-induced atrophy.[4] However, increased hepatic synthesis of ALR and powerful mitogens, such as hepatocyte growth factor (HGF) and TGFα (transforming growth factor α), after portacaval shunt in rats indicated that first pass of gastrointestinal-derived growth factors is critical for liver cell size maintenance and function.[45] ALR also protects the liver from galacosamine-,[4] carbon tetrachloride (CCl_4)-,[46] H_2O_2-,[47] ethanol-,[48] and acetaminophen-mediated[49] acute injury. Transplantation of liver epithelial progenitor cells overexpressing ALR was shown to mitigate CCl_4-induced liver damage and mortality, whereas ALR silencing had the opposite effects.[50] ALR was also shown to protect human hepatocytes in primary culture from pro-apoptotic agents.[51]

Accumulation of toxic bile acids during cholestasis causes oxidative stress and death of hepatocytes. Reduced hepatic ALR levels in human cholestatic liver disease (presumably via bile acid-induced SHP activation and suppression of *ALR* promoter activity),[39] and inhibition of glycochenodeoxycholic acid-induced apoptosis in s-ALR-transfected HepG2 cells[52] indicate the importance of this cytosolic form in cell survival. In contrast, glycochenodeoxycholic acid-mediated apoptosis is not affected by l-ALR-transfected Huh7 cells.[53] Interestingly, ALR levels are increased in primary biliary cholangitis, sclerosing cholangitis and cholangiocarcinoma.[6] Mechanisms underlying the variable expression of the *ALR* gene and their implications in progression of cholestatic liver disease remain to be elucidated.

The ALR receptor has been identified in rat hepatocytes, and binding of s-ALR promotes DNA synthesis with similar potency as the powerful hepatocyte growth factors HGF, TNFα and EGF.[54] The direct effect of s-ALR on hepatocytes is reported to be mediated by EGFR phosphorylation and subsequent activation of AP1,[55] as well as polyamine synthesis via c-Myc activation.[56] Binding of l-ALR to Jun activation domain-binding protein 1 (JAB1) also promotes AP1 transcriptional activity.[57,58] These findings suggest that both ALR forms can stimulate liver regeneration.

Importance of ALR in mitochondrial integrity and function

Mutations in the *scERV1* gene or depletion of scERV1 protein caused loss of the inner mitochondrial membrane and eventually the entire organelle, indicating the critical importance of ERV1 in mitochondrial integrity, survival and function.[59,60] Most of the mitochondrial proteins are synthesised in the cytosol and transported into the mitochondria as precursors, aided by the TOM (translocase of the outer membrane) complex.

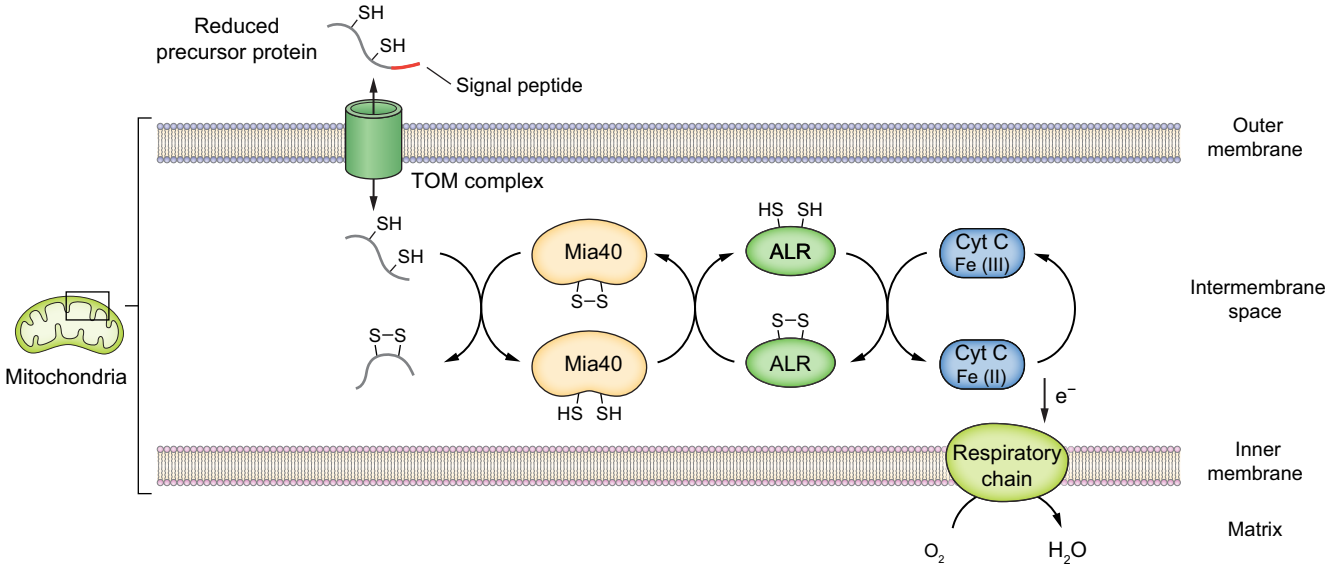

Fig. 2. ALR/Mia40 mitochondrial disulphide relay system. Newly synthesised proteins in the cytosolic compartment are translocated into mitochondrial intermembrane space through the TOM channel. The imported proteins are oxidised by Mia40 allowing for their proper folding. Following oxidation by sulfhydryl oxidase activity of Erv1/ALR, Mia40 re-enters the cycle to introduce a disulphide bond in incoming proteins. ALR is subsequently re-oxidised by donating electrons to cytochrome c for the reaction involving conversion of oxygen to water by cytochrome c oxidase. ALR, augmenter of liver regeneration; Mia40, mitochondrial intermembrane space import and assembly protein 40 kDa; TOM, translocase of the outer membrane.

Appropriate oxidative folding of several of these proteins is essential for their functions and is catalysed by the Mia40 (mitochondrial intermembrane space import and assembly protein 40 kDA)/ALR-sulfhydryl relay system[61–64] (Fig. 2). Mia40, with its redox-active cysteine-proline-cysteine disulphide bond, oxidises cysteine residues of the imported polypeptides; these stably folded proteins are prevented from transport through the outer membrane.[65,66] Reduced Mia40 is re-oxidised by ALR (about 70% of Mia40 is in an oxidised state) and can then introduce disulphide bonds into the newly imported polypeptides.[67] Two essential redox-active cysteine-x-x-cysteine pairs in ALR shuttle electrons from Mia40 to FAD. ALR can be directly re-oxidised by oxygen *in vitro* in a reaction yielding H_2O_2. *In vivo*, ALR is re-oxidised by passing its electrons through FAD to cytochrome c of the respiratory chain; these electrons are then accepted by molecular oxygen to produce water, thus preventing generation of H_2O_2 in the intermembrane space.[68–70]

The presence of ALR in excess of Mia40[71] indicates that its role in mitochondria may extend beyond Mia40 reoxidation. Indeed, mitochondrial ALR plays an essential role in iron homeostasis by catalysing Fe/S maturation of cytosolic proteins.[72] Mechanistically, the Mia40/ALR pathway has been shown to facilitate import of ABCB8 (ATP-binding cassette-B8), an inner mitochondrial membrane protein necessary for cytoplasmic Fe/S cluster maturation.[73] The pathophysiological implication of ALR as a regulator of iron homeostasis is evidenced by excessive iron accumulation in the liver of hepatocyte-specific ALR-deficient mice upon alcohol consumption.[18] Clinically, the importance of ALR in the mitochondrial sulfhydryl relay system is exemplified by mitochondriopathy, decreased activity of respiratory complexes I, II and IV, congenital cataract, muscular hypotonia and developmental delay observed in patients with a rare (R194H) mutation in the *ALR* gene.[74,75] The mutation reduces stability of the ALR protein, as well as the expression and activity of cytochrome oxidase. The R194H mutation also causes defective accumulation of Mia40 in mitochondria suggesting that ALR regulates Mia40 localisation.[76]

Another mechanism by which ALR might be important in mitochondrial function is by regulating TFAM, a highly conserved nuclear-encoded DNA-binding protein.[77–79] TFAM is critical for stabilisation and transcription of mitochondrial DNA (mtDNA).[80,81] Administration of recombinant ALR to normal rats time-dependently increases TFAM expression.[43] Genetic ablation of *ALR* from hepatocytes reduces TFAM expression and ATP content, which recover as ALR levels increase.[14] In line with this observation, ALR-knockdown in mice reduces expression of TFAM as well as PGC-1α (peroxisome proliferator-activated receptor-γ coactivator-1α), impairs mitochondrial biogenesis, and delays liver regeneration after partial hepatectomy.[82] In a rat model of obstructive jaundice, administration of human ALR led to increased expression of TFAM and nuclear respiratory factor 1, mtDNA damage repair and improvement of mitochondrial functions.[83] Mitochondrial fission (via phosphorylation of dynamin-related protein 1) upon knockdown of ALR

Key point

ALR maintains lipid homeostasis by regulating expression of lipogenic and lipolytic enzymes.

from liver epithelial progenitor cells also demonstrates its importance in mitochondrial survival.[50]

The mitochondrial electron transport chain is the site of ATP and reactive oxygen species (ROS) generation, as few electrons interact directly with oxygen to form superoxide anion ($O_2^{\cdot -}$) and H_2O_2.[84] ROS damage mtDNA, interact with lipids and proteins to form peroxidation products and cause cell death. ALR depletion *in vivo* and *in vitro* increases oxidative stress and causes mtDNA damage.[14] Forced ALR deficiency-induced oxidative stress and lipid accumulation in hepatocytes is reversed by ALR treatment.[15] Of note, the Mia40/ALR-sulfhydryl relay system was shown to introduce functional protein folding in superoxide dismutase 1.[85–87] Increased ALR expression also imparts protection against irradiation-induced mitochondrial and cellular damage by increasing mitochondrial membrane potential, inhibiting cytochrome c release and preventing ATP loss.[44]

Role of ALR in NAFLD

Considering the importance of ALR in mitochondrial biogenesis, survival and function, a deficiency of, or an abnormality in, the ALR protein might be a predisposing condition in the development of NASH, the aggressive form of NAFLD. NASH is characterised by steatosis, hepatocyte ballooning and injury, inflammation, and pericellular fibrosis.[88] While the majority of patients with NAFLD have simple steatosis (NAFL), up to 30% develop NASH and may progress to cirrhosis, a fertile environment for HCC.[20–22] Obesity, sedentary lifestyle, type 2 diabetes mellitus/insulin resistance, altered gut microbiota, and genetic and environmental factors are all considered as contributors to NAFLD/NASH and NASH-induced cirrhosis.[21,89] Despite extensive clinical and experimental research, there is still a significant knowledge gap regarding the pathogenesis and progression of NAFLD, for which no pharmacological treatment has been approved.[21,90]

Key point

Genetic ablation of *Alr* in mice leads to robust steatosis and cell death followed by "lean" NASH-like progression to hepatocellular carcinoma.

Ultrastructural mitochondrial lesions, increased production of ROS and lipid peroxidation (due to decreased activity of respiratory chain complexes), decreased fatty acid β-oxidation, and reduced ability to resynthesise ATP have all been observed in patients with NASH.[23–26] However, mechanisms underlying deterioration of mitochondrial structure and function are not completely understood. It should be noted that hepatic mitochondria of obese individuals with or without NAFL exhibit an increased respiration rate, but this adaptation is lost in those with NASH who exhibit a significantly reduced rate of respiration in association with increased oxidative stress and DNA damage.[91,92] It will be important to elucidate whether this transition occurs as patients progress from NAFL to NASH, or if patients who progress to NASH are distinct from those with NAFL who do not develop NASH.

Hepatocyte-specific *Alr*-knockout (*Alr*-H-KO) mice were generated to investigate ALR's role in liver physiology. *Alr*-H-KO mice showed severe mitochondriopathy (degenerating or enlarged mitochondria with loss of cristae, and defect at complex II of the electron transport chain), and ATP depletion due to significant loss of ALR between 1 and 2 weeks postpartum.[14] There was robust mixed macro- and microvesicular steatosis, excessive liver cell death and pericellular fibrosis.[14] These pathologies led to strong ductular reaction, regeneration of hepatocytes from the surviving cells, as well as from cells of the biliary compartment at 4 weeks, and regression of steatosis. It was observed that the surviving/regenerating cells expressed ALR, albeit at significantly lower magnitude than cells in the wild-type (WT) mouse liver, but there was continued inflammation and modest fibrosis. Eventually, nearly 70% of *Alr*-H-KO mice developed liver tumours (60% being HCC) by 1 year when their ALR expression was similar to the WT mice.[14] In this regard, hepatic ALR levels were found to be greater than normal in HCC.[6] Interestingly, hepatic ALR levels, measured via western blot analysis or ELISA (pg/mg protein or DNA), are lower than normal in NASH-induced cirrhosis.[14,16] Since cells from the biliary compartment seem to transform into ALR-expressing hepatocytes following pronounced apoptosis at 2 weeks postpartum, they are the likely source of malignancy. Although NAFLD is generally associated with central obesity, patients with a normal body mass index developing "lean" NASH have been identified.[20] Furthermore, based on routine cancer screening, 35–50% of HCC cases occurring in patients with NASH arise in the absence of cirrhosis.[93,94] *Alr*-H-KO mice could be used to study lean NASH and NASH-induced HCC in the absence of cirrhosis, since they do not become overweight/obese or develop cirrhosis.

Hepatic ALR expression is lower than normal in NAFL,[19] NASH and NASH-cirrhosis.[16,19] Our analysis showed physiological serum ALR concentrations in the 32-380 pg/ml range (n = 27), indicating inter-individual variability; ALR concentrations were significantly lower in NASH (0-336 pg/ml; p <0.05 *vs.* Control; n = 25) and NASH-cirrhosis (24-380 pg/ml; p <0.05 *vs.* Control; n = 22). In contrast, serum ALR increases in acute, chronic and fulminant hepatitis due to hepatitis virus A, B or C infection.[95,96] Because the liver is the primary source of circulating ALR,[11] the increase may be due to ALR released from injured/dying hepatocytes.[10] However, normal serum ALR concentrations reported in these studies ranged from 3.0±1.55 pg/ml[95] to 3.77±1.55 ng/ml.[96] Discrepancies in these values may be due to differential specificity of the ALR antibodies used and differences in assay procedures. Thus, a more comprehensive investigation is required to ascertain the serum ALR range in NAFL, NASH and NASH-cirrhosis. Such research might lead to determination of serum ALR concentrations predictive or diagnostic of NASH. Similar or greater hepatic ALR levels in some patients with NASH-

cirrhosis compared to healthy individuals[16] indicate heterogeneity in the disease. More intense staining for ALR was also observed in some regenerating liver cell nodules of patients with NASH-cirrhosis.[16]

The nuclear localisation of ALR suggests that it may also have a nuclear function. Although binding of ALR to JAB1 increases AP1-activity,[32,57,58] how it regulates the expression of proteins involved in lipid homeostasis is unclear. The loss of ALR *in vivo* and *in vitro* reduces the expression of carnitine palmitoyl transferase a (CPT1a), sterol regulatory element-binding protein (SREBP)1c, peroxisome proliferator-activated receptor-α (PPARα), peroxisomal membrane protein 70 (PMP70) and acyl-CoA oxidase 1 (ACOX1).[14,15] Forced overexpression of ALR causes upregulation of CPT1a in steatotic hepatocytes.[19,97] The mechanism of ALR depletion-induced dysregulated lipid homeostasis appears to involve altered expression of several microRNAs (miRNAs) with binding sequences on mRNAs encoding CPT1a, SREBP1c, PPARα, PMP70 and ACOX1.[15] In fact, expression of several miRNAs implicated in human NAFL and NASH are similarly altered in *Alr*-H-KO mice between 1 (beginning of steatosis) and 4 (NASH-like phenotype) weeks postpartum.[15] Increased miR-540 (miR-6801 in humans) was found to affect the expression of CPT1a, SREBP1c, PPARα, PMP70 and ACOX1, and treatment with anti-miR-540 or recombinant ALR between 1 and 2 weeks mitigated steatosis and pericellular fibrosis in *Alr*-H-KO mice.[15] Although mitochondrial injury-related oxidative stress might be a causal factor for the altered expression of miRNAs and mRNAs, whether ALR influences the binding and activity of nuclear transcription factors responsible for the expression of miRNAs remains to be determined.

The *GFER* (*ALR*) gene contains several single nucleotide polymorphisms, some of which are pathogenic (https://www.ncbi.nlm.nih.gov/snp/) (Table 1). It is noteworthy that children receiving the hypofunctional *GFER* allele (R194H mutation; rs121908192) from both healthy heterozygous parents develop severe mitochondriopathy, progressive myopathy and partial combined respiratory chain deficiency, congenital cataract, sensorineural hearing loss, and developmental delay.[74] Other investigations found that homozygous or heterozygous mutations in *GFER* (rs121908192; rs1555486560; rs1597063051; rs1597063303; rs771809901) in the same patient induced similar congenital progressive multiorgan pathologies.[75,98] Di Fonzo performed ultrasound in only 1 patient and did not report liver involvement.[74] However, ultrasound evaluation for steatosis is only accurate when >25% of the liver is affected, and biopsy is essential to diagnose steatohepatitis. Importantly, liver biopsy of a patient with compound heterozygous mutation (frameshift variants c.219delC [p.(Cys74Alafs*76)] and [c.259-25_259-24delCA]) showed mitochondrial damage, and centrolobular and portal fibrosclerosis, and electron microscopy revealed a number of pleiomorphic mitochondria containing paracrystalline inclusions.[75] Very little is known about these mutations in acute and chronic liver diseases. Because subnormal ALR levels are observed in human NAFL, NASH and NASH-induced cirrhosis,[14,16,19] it is likely that individuals with inherent ALR deficiency or dysfunction might be predisposed to develop aggressive NASH. Alternatively, steatosis-induced downregulation of ALR might be an important contributing factor to the severity of disease progression.

Key point

ALR deficiency promotes experimental high-fat diet-induced NAFLD/NASH, and its levels are reduced in human NASH and NASH-cirrhosis.

Hepatocyte-specific *Alr*-heterozygous (*Alr*-H-HET) mice, which demonstrate normal development and function were investigated to examine whether ALR deficiency is a risk factor for NAFLD/NASH.[16] *De novo* lipogenesis and reduced mitochondrial β-oxidation contribute to steatosis in NAFLD.[21] The basal expression of SREBP1c, acetyl-CoA carboxylase (ACACA), fatty acid synthase (FASN) and SREBP2 was found to be somewhat higher in *Alr*-H-HET compared to WT mice.[16] A high-fat high-carbohydrate (HF/HC) diet induced greater obesity and hepatic steatosis (increased *de novo* lipogenesis and depressed lipolysis) in *Alr*-H-HET mice than in WT mice, with relevant changes in the expression of related enzymes (SREBP1c, ACACA, and FASN).[16] HF/HC-fed *Alr*-H-HET mice had greater hepatic levels of free fatty acids, triglycerides and cholesterol than their WT counterparts.[16] A study published concurrently also reported increased SREBP2 expression and cholesterol accumulation in global *Alr*-HET mice.[99] The authors went on to show that inactivation of AMPK (AMP-activated protein kinase) leads to increased cholesterol in high-fat diet-fed *Alr*-HET mice. Accumulation of free cholesterol induces endoplasmic reticulum stress and mitochondrial injury by inhibiting glutathione transport. Furthermore, mitochondrial accumulation of cholesterol causes JNK activation and subsequent apoptosis/necrosis. Thus, based on the cholesterol accumulation observed in liver biopsies of patients with NASH, which correlates with severity of NASH and NASH-fibrosis, it is proposed that increased accumulation of cholesterol is a major contributor to ongoing lipotoxicity in experimental and human NASH.[100,101] These findings provide further support for the impact of ALR deficiency on NASH development, and are supported by the greater magnitude of reduction in hepatic ALR in HF/HC diet-fed *Alr*-H-HET mice than WT mice.[16] Furthermore, overexpression of ALR via plasmid transfection mitigated high-fat diet-induced hepatic steatosis by increasing CPT1a activity.[97] Also, forced downregulation of ALR increased lipid accumulation and lipotoxicity in primary hepatocytes,[15] and exogenous ALR reduced these effects.[19]

Key point

Several single nucleotide polymorphisms found in the human *ALR* gene cause developmental delay, progressive multiorgan pathologies and death.

HF/HC-fed *Alr*-H-HET mice had increased inflammation in the liver (greater incidence of

Table 1. Pathogenic SNPs in the *GFER* gene.

Position	dbSNP	Variant type:	Gene: consequence/ protein change	Clinical significance	ClinVar accession
chr16:1985991 (GRCh38.p13)	rs121908192 [*Homo sapiens*]	Variant type: SNV	GFER: missense variant NP_005253.3:p.Arg194His	Myopathy, mitochondrial progressive, with congenital cataract, hearing loss, and developmental delay	RCV000009228.8
Exon -3		alleles: G>A	R (Arg) > H (His) R [CGC] > H [CAC]	Inborn genetic diseases	RCV000624237.1
				Disease name: ND	RCV000199876.1
chr16:1985996 (GRCh38.p13)	rs370475970 [*Homo sapiens*]	Variant type: SNV	GFER: missense variant	Mitochondrial diseases	RCV000508691.1
Exon -3		alleles: C>T	NP_005253.3:p.Arg196Cys R [CGC] > C [TGC]	Myopathy, mitochondrial progressive, with congenital cataract, hearing loss, and developmental delay	RCV000709773.1
chr16:1985976 (GRCh38.p13)	rs373135339 [*Homo sapiens*]	Variant type: SNV	GFER: stop gained missense variant	Inborn genetic diseases	RCV000622535.1
Exon -3		alleles: C>G,T	NP_005253.3:p.Ser189Ter S [TCA] > * [TGA] NP_005253.3:p.Ser189Leu S [TCA] > L [TTA]		
chr16:1984861 (GRCh38.p13)	rs771809901 [*Homo sapiens*]	Variant type: SNV	GFER: stop gained NP_005253.3:p.Gln125Ter	Myopathy, mitochondrial progressive, with congenital cataract, hearing loss, and developmental delay	RCV001254645.1
Exon 2		alleles: C>T	Q (Gln) > * (Ter) Q [CAG] > * [TAG]	Disease name: ND	RCV000199819.1
chr16:1984415-1984417 (GRCh38.p13)	rs863224028 [*Homo sapiens*]	Variant type: indel	GFER: frameshift variant	Mitochondrial diseases	RCV000508880.1
		alleles: C>-(delC)	NP_005253.3:p.Arg67fs	Myopathy, mitochondrial progressive, with congenital cataract, hearing loss, and developmental delay (pathogenic/likely pathogenic)	RCV001270124.2
Exon 1	rs1288218335 and rs747241374 have been merged into rs863224028		R (Arg) > G (Gly)	Disease name: ND (likely pathogenic)	RCV000200750.1
chr16:1984433-1984435 (GRCh38.p13)	rs1555486560 [*Homo sapiens*]	Variant type: indel alleles: delG	GFER: frameshift variant NP_005253.3:p.Ala73fs A (Ala) > P (Pro)	Myopathy, mitochondrial progressive, with congenital cataract, hearing loss, and developmental delay	RCV000679993.2
Exon 1			A [GCC] > P [CC]	Disease name: ND (likely pathogenic)	RCV000676337.1
chr16: 1985985 (GRCh38) chr16: 2035986 (GRCh37) Exon 3	Accession: SCV001760360.1 *[Homo sapiens]* Submitted: (Jul 15, 2021)	Variant type: SNV alleles: A>G	GFER: missense NP_005253.3:p.Asp192Gly D192G A>G	Myopathy, mitochondrial progressive, with congenital cataract, hearing loss, and developmental delay	RCV001542783.1
chr16:1984436-1984437 (GRCh38.p13)	rs1597063051 [*Homo sapiens*]	Variant type: deletion	GFER: frameshift variant NP_005253.3:p.Cys74fs A (Ala) > A (Ala) A [GCC] > A [GC]	Myopathy, mitochondrial progressive, with congenital cataract and developmental delay	RCV000824904.2
Exon 1					

dbSNP, single nucleotide polymorphism database; Indel, insertion and deletion; ND, not determined; SNV, single nucleotide variation.

TNFα-, IL-6- and IL17-producing cells and lower incidence of FoxP3+ immunosuppressive regulatory T cells) and in white adipose tissue. This model is relevant to human NASH since both female and male mice developed hepatocyte injury, inflammation, stellate cell activation, and fibrosis. *Alr*-H-KO mice with underlying modest inflammation and fibrosis are resistant to HF/HC-induced obesity and hepatic steatosis but progressed to cirrhosis.[16] These findings are akin to human NASH-induced cirrhosis in which steatosis is absent or minimal.[16,102] The importance of ALR deficiency in steatohepatitis is exemplified by lower hepatic ALR in human alcohol-related cirrhosis and the occurrence of alcohol-induced cirrhosis within 4 weeks in mice fed the Lieber de Carli diet (while WT mice fed the same diet showed only modest steatosis at the same timepoint).[18] A similar predisposition to NAFLD or alcohol-related liver disease is likely in humans with 1 functional *ALR* allele.[74,75]

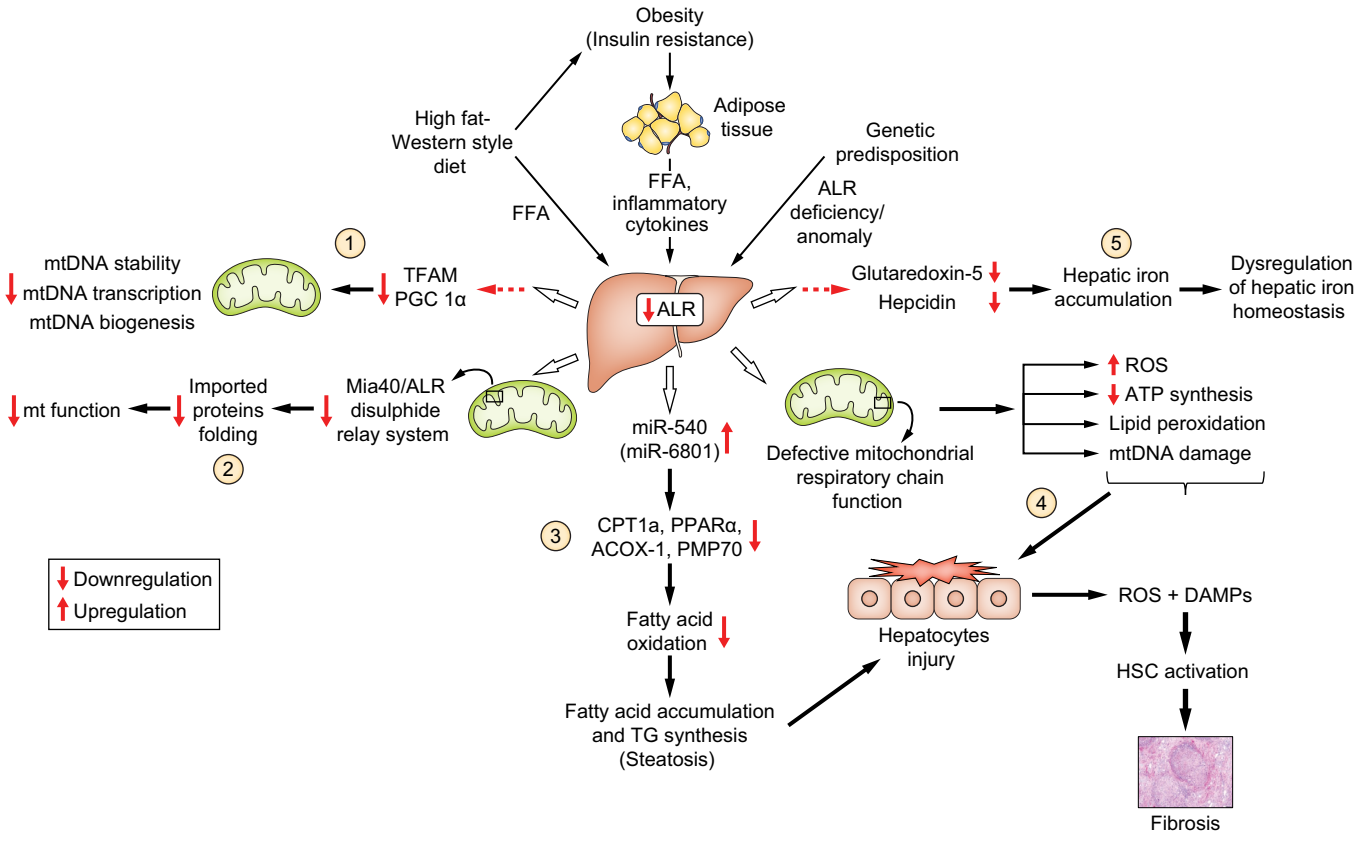

Fig. 3. Putative mechanisms of NAFLD due to ALR deficiency. (1) High-fat Western-style diet causes obesity, insulin resistance and hepatic steatosis. FFAs and inflammatory cytokines released from adipose tissue further contribute to steatosis, which downregulates ALR expression. Inherent deficiency or dysfunction of ALR may also predispose the liver to abnormal lipid homeostasis causing increased steatosis. (2) Decreased hepatic ALR may cause downregulation of TFAM and PGC1α expression resulting in reduced mtDNA stability, transcription and biogenesis. ALR deficiency reduces the activity of the Mia40/ALR disulphide relay system causing disrupted oxidative folding of imported proteins leading to inhibition of mitochondrial function. (3) ALR deficiency increases miR-540, which disrupts mitochondrial and peroxisomal lipid homeostasis by inhibiting expression of CPT1a, PPARα, ACOS-1 and PMP70, thus increasing steatosis. (4) ALR deficiency disrupts mitochondrial respiratory chain activity causing increased ROS generation, reduced ATP synthesis, lipid peroxidation and mtDNA damage. This and FFA toxicity cause hepatocyte injury and consequent release of ROS and DAMPs, which promote activation of hepatic stellate cells to a fibrogenic phenotype and fibrosis development. (5) Reduced ALR in steatohepatitis may also cause dysregulation of iron homeostasis through reduced expression of GLRX5 and hepcidin leading to iron accumulation and toxicity. Dashed red arrows indicate that the mechanisms of those pathways have not been elucidated. ACOX-1, acyl-coenzyme A oxidase 1; ALR, augmenter of liver regeneration; CPT1a, carnitine palmitoyl transferase I; DAMPs, damage-associated molecular patterns; FFAs, free fatty acids; GLRX5, glutaredoxin-5; HSC, hepatic stellate cell; mt, mitochondrial; PMP70, peroxisomal membrane protein 70; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; PPARα, peroxisome proliferator-activated receptor-α; ROS, reactive oxygen species; TFAM, mitochondrial transcription factor A; TG, triglyceride.

Paracrystalline inclusions, loss of cristae, and multilamellar membranes in mitochondria of patients with homozygous (R194H) mutations,[74,75] abnormalities also recognised in NAFLD,[103] all raise the possibility that ALR dysfunction might be critically involved in mitochondriopathy in NASH. Fig. 3 summarises putative pathways of NAFLD involving deficiency or dysfunction of ALR.

ALR in alcohol-induced liver injury

Mitochondrial dysfunction is also implicated in alcohol-induced liver injury, another common cause of chronic liver disease worldwide.[24,104] Alcohol-related liver disease (ALD) also includes a spectrum of conditions such as simple steatosis, hepatitis, fibrosis, cirrhosis, and HCC.[105] Although heavy drinkers are prone to develop hepatic steatosis, about 20-40% progress to alcohol-related steatohepatitis and 8-20% advance to cirrhosis.[106] It is postulated that genetic and environmental factors are drivers of ALD from simple steatosis to fibrosis and cirrhosis.[104,107] Like humans, most animal models are resistant to more aggressive ALD, and steatosis is readily reversed upon termination of alcohol ingestion. The Lieber Di Carli liquid alcohol diet, which has been used extensively to study ALD in mice, caused steatosis in control mice, but promoted aggressive liver disease leading to cirrhosis, accompanied by reduced expression of alcohol dehydrogenases-1 and aldehyde dehydrogenases-1, in ALR-deficient mice.[18] There was also significant mitochondrial damage and iron accumulation (lower glutaredoxin-5 and hepcidin expression) in alcohol-fed ALR-deficient mice. The clinical significance of these findings is indicated

by significantly lower hepatic ALR expression in patients with alcohol-related cirrhosis.[14,18] However, *Alr*-H-KO mice already have underlying modest oxidative stress, inflammation and fibrosis that are accelerated/augmented by alcohol, as demonstrated by further increases in oxidative stress, robust lipid peroxidation and mtDNA damage. Such underlying conditions in humans are likely a prerequisite for aggressive ALD. ALR was also shown to protect mice from alcohol-induced acute liver injury by promoting autophagy through repression of mTOR (mammalian target of rapamycin).[108] Furthermore, overexpression of ALR improved mitochondrial membrane potential and increased ATP levels in alcohol-treated HepG2 cells.[108] NRF2, which upregulates ALR during oxidative stress, is protective against alcohol-induced hepatic and pancreatic damage.[109]

Key point

ALR deficiency or dysfunction may be an important risk factor for NASH.

Conclusions and future prospects

Despite being an evolutionally conserved fundamental life protein with varied functions, understanding of the role of ALR in physiology and pathophysiology has been inadequate. ALR is critically important for mitochondrial biogenesis, protein folding (Mia40-sulfhydryl relay system), and respiratory chain activity, disruption of which is implicated in the pathogenesis and progression of both NAFLD and ALD. *In vivo* and *in vitro* studies of ALR-knockdown and hepatocyte-specific ALR deficiency have provided crucial evidence of ALR's role in lipid homeostasis and in promoting the expression of several genes, including those involved in alcohol and iron metabolism. The clinical significance of ALR in NASH and ASH is inferred from its lower hepatic concentration in human NASH- and ASH-cirrhosis. Because of its lack of a DNA-binding sequence, ALR may not be directly involved in gene transcription but may act as a promoter or suppressor of certain transcription factors. In this regard, ALR immunoprecipitates with TFAM (unpublished observation), and deficiency of ALR downregulates TFAM expression. Several pathogenic single nucleotide polymorphisms are found to cause severe mitochondrial damage and progressive multiorgan disease in humans. Liver biopsy of 1 patient with mutations in both *ALR* alleles showed hepatic mitochondrial damage and fibrosis. Thus, humans with heterozygous mutations in the *ALR* gene could be predisposed to chronic liver diseases such as NASH and ASH. Future investigations to further delineate mechanisms by which ALR deficiency or dysfunction promotes NAFLD or ALD progression will be important.

Abbreviations

ACACA, acetyl-CoA carboxylase alpha; ACOX1, acyl-CoA oxidase 1; ALD, alcohol-related liver disease; ALR, augmenter of liver regeneration; CCl_4, carbon tetrachloride; C/EBPβ, CCAAT/enhancer binding proteins; CPT1a, carnitine palmitoyl transferase I a; Egr-1, Early growth response protein-1; ERV1, essential for respiration and vegetative growth-1; ESLD, end-stage liver disease; FAD, flavin adenine dinucleotide; FASN, fatty acid synthase; FoxA2, forkhead box A2; HCC, hepatocellular carcinoma; HF/HC, high-fat high carbohydrate; HNF4α, hepatocyte nuclear factor 4 alpha; l-ALR, long form ALR; IMS, intermembrane space; JAB1, Jun activation domain-binding protein 1; Mia40, mitochondrial IMS import and assembly protein 40 kDa; miRNA, microRNA; mtDNA, mitochondrial DNA; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NRF2, nuclear factor erythroid 2-related factor 2; nt, nucleotide; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1-alpha; PMP70, peroxisomal membrane protein 70; PPARα, peroxisome proliferator-activated receptor alpha; rALR, recombinant ALR; ROS, reactive oxygen species; s-ALR, short form ALR, scERV1, *Saccharomyces cerevisiae* essential for respiration and vegetative growth-1; SHP, small heterodimer partner; SREBP, sterol regulatory element-binding protein; TFAM, mitochondrial transcription factor A.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

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Authors' contributions

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Supplementary data

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Author names in bold designate shared co-first authorship

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