

# World Journal of *Gastroenterology*

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Baishideng Publishing Group Inc  
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Telephone: +1-925-2238242  
Fax: +1-925-2238243  
E-mail: [editorialoffice@wjgnet.com](mailto:editorialoffice@wjgnet.com)  
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## Interleukin 12/interleukin 23 pathway: Biological basis and therapeutic effect in patients with Crohn's disease

Ioanna Aggeletopoulou, Stelios F Assimakopoulos, Christos Konstantakis, Christos Triantos

Ioanna Aggeletopoulou, Christos Konstantakis, Christos Triantos, Division of Gastroenterology, Department of Internal Medicine, Medical School, University of Patras, Patras 26504, Greece

Stelios F Assimakopoulos, Department of Internal Medicine, University Hospital of Patras, Patras 26504, Greece

ORCID number: Ioanna Aggeletopoulou (0000-0003-4489-1485); Stelios F Assimakopoulos (0000-0002-6901-3681); Christos Konstantakis (0000-0001-5834-9182); Christos Triantos (0000-0003-3094-8209).

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**Correspondence to:** Christos Triantos, PhD, Assistant Professor in Internal Medicine and Gastroenterology, Department of Gastroenterology, University Hospital of Patras, D. Stamatopoulou 4, Patras 26504, Greece. [chtriantos@upatras.gr](mailto:chtriantos@upatras.gr)  
Telephone: +30-69-72894651  
Fax: +30-26-10625382

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### Abstract

Considering that both innate and adaptive immune responses are involved in the pathogenesis of Crohn's disease (CD), novel therapeutic options have significantly been developed. Biological agents represent an important addition to the conventional treatments for immuno-inflammatory conditions, acting as antagonists of adhesion molecules or various inflammatory cytokines. The interleukin 12 (IL-12)/IL-23 common pathway has been found to play a determinant role in the induction of inflammation in adaptive immune responses. In particular, IL-23 promotes the differentiation of naïve T helper cells into Th17 phenotype with the concomitant secretion of several inflammatory cytokines such as IL-17 and IL-22, whereas IL-12 induces the Th1 polarization and production of critical cytokines such as interferon- $\gamma$  and tumor necrosis factor. Nowadays, there is increased interest regarding the role of IL-23 as a therapeutic target of CD through the blockage of IL-23 mediated pathways. In this editorial, we focus on the role of IL-12/IL-23 pathway in the regulation of mucosal immunity and in the induction and maintenance of chronic inflammation. In parallel, we critically discuss the available data regarding the therapeutic effect of the IL-12/IL-23 inhibitors and especially of ustekinumab, a human monoclonal antibody which has been recently approved by the United States Food and Drug Administration for the management of moderate-to-severe CD and its potential to be used as first-line therapy in everyday clinical practice.

**Key words:** Crohn's disease; Interleukin 12; Interleukin



23; Monoclonal antibodies; Ustekinumab; Biological agents; Interleukin 12/interleukin 23 blockade

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**Core tip:** The therapeutic management of Crohn's disease patients not responding to treatment with anti-tumor necrosis factor agents remains a clinical challenge. Recently, there has been increased evidence regarding the development of new drugs with alternative mechanisms of action. Interleukin (IL)-12 and IL-23 are important cytokines which are involved in the adaptive immune responses and their common pathway has been found to play a determinant role in the induction of inflammation. Clinical trials have assessed the therapeutic effect of an IL-12/IL-23 inhibitor (ustekinumab), demonstrating rapid clinical effect with a safety profile. Further studies are needed to elucidate its potential role as first-line therapy in Crohn's disease.

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## INTRODUCTION

Crohn's disease (CD) is an immune-mediated inflammatory disorder characterized by chronic relapsing inflammation in different segments of the gastrointestinal tract. Although the typical preferential regions of involvement are the distal ileum, the ileocaecal region, the colon and the perianal region, extra-intestinal manifestations are not uncommon<sup>[1]</sup>. The etiology of this disease is not yet fully understood. However, it is currently considered that genetic and environmental factors, impaired immune regulation, gut barrier dysfunction and changes in the intestinal microbiome are involved in the pathogenesis and development of this condition<sup>[2-4]</sup>. CD treatment is generally individualized and is associated with several factors including disease phenotype, disease severity, affected region, and related luminal or extraluminal complications. The treatment strategy is mainly classified into two stages: (1) Appropriate treatment of the acute flare aiming to induce remission (2) maintenance of remission<sup>[5]</sup>. Until recently, the initial choice of treatment has focused on long-term use of corticosteroids and immunosuppressants like thiopurines and methotrexate for the induction and the maintenance of remission, respectively<sup>[6-8]</sup>. During the last years, therapeutic options have significantly benefited from the introduction of biological agents,

which became the mainstay of moderate to severe CD treatment, using monoclonal antibodies targeting tumor necrosis factor (TNF)<sup>[9-11]</sup> or adhesion molecules (integrins)<sup>[12,13]</sup>. However, a significant proportion of patients (about 30%) will not respond adequately to induction therapy with TNF inhibitors. Furthermore, another subgroup of patients that achieve initial (short-term) response, run a risk of secondary loss which occurs in approximately 40% of patients<sup>[14,15]</sup>. The main causes of secondary failure are non-compliance to anti-TNF treatment, drug immunogenicity and non-immune clearance of anti-TNF or the persistence of inflammatory activity in spite of sufficient anti-TNF levels<sup>[16]</sup>. This latter clinical scenario is usually performed by switching to another class of biological agents<sup>[16]</sup>. Moreover, the humanized anti- $\alpha 4\beta 7$  integrin antibody that has been recently introduced in clinical practice, has displayed efficacy on the induction and maintenance of remission in moderate-to-severe refractory CD patients; however, safety concerns have been raised due to rare but possible adverse events<sup>[17]</sup>.

Current data suggest that the initiation and perpetuation of inflammation in CD are associated with a disruption in the balance among the intestinal epithelium, the commensal microbiota and the innate immune response<sup>[18]</sup>. This condition is maintained by the presence of defects in the intestinal wall, environmental factors, genetic predisposition and dysfunction in regulatory mechanisms, which in turn lead to the release of an array of cytokines that promote the inflammatory immune response<sup>[18]</sup>.

Taking into consideration the adverse events resulted from previous treatments regimens, target tailored treatment options that aim at specific pathways of inflammation have emerged. CD is characterized by dysfunction in both innate and adaptive immune responses. Disturbances in adaptive immune response are closely related to tissue damage, mainly driven by interleukin (IL)-12 and IL-23<sup>[4]</sup>. Therefore, inhibitors of IL-12/IL-23 and specific inhibitors of IL-23 have been developed for the management of CD. In this editorial, we focus on the role of IL-12/IL-23 pathway in the modulation of mucosal immunity and in the induction and maintenance of remission of the associated chronic inflammation of the intestinal epithelium. Moreover, we critically discuss the therapeutic effects of the IL-12/IL-23 blocker in patients with CD and its potential position as first-line therapy in everyday clinical practice.

## IL-12 AND IL-23 ROLE IN CD/ MECHANISM OF ACTION

Both innate and adaptive immune responses are involved in the pathogenesis of CD. Adaptive immunity is provided by resident and recruited cells, including mucosal B cells which produce the secretory immunoglobulins A and G, T cells subpopulations and

especially T helper 1 (Th1), Th17, or Th2 cells, and T and B regulatory cells<sup>[41]</sup>. Th1 phenotype is induced by microbes which in turn activate the excretion of interferon (IFN)- $\gamma$  and IL-12p40 through the signal transducer and activator of transcription 1 (STAT1), T-box factor 21 (TBX21) and STAT4 signaling pathways<sup>[19]</sup>. CD seems to be driven by a Th1-mediated pathology, with an increased synthesis of IFN- $\gamma$ <sup>[20]</sup>. In parallel, inflamed intestinal mucosa is infiltrated by Th17 cells with a concomitant production of IL-17 cytokine<sup>[21]</sup>. Th17 lineage commitment is directed by transforming growth factor beta (TGF- $\beta$ ) in the presence of a proinflammatory environment, and IL-23 is related to the expansion and maintenance of Th17 cells<sup>[22]</sup>. Moreover, CD is characterized by an increased production of IL-12, the major Th1-stimulating factor<sup>[23,24]</sup>.

IL-12 family includes IL-12, IL-2, IL-35 and IL-27, key mediators of inflammatory response<sup>[25]</sup>. IL-12 is a heterodimeric cytokine comprising of two covalently linked subunits (p40 and p35) and is mainly produced by activated phagocytic cells [monocytes/macrophages, neutrophils, dendritic cells (DCs)] in response to bacterial stimulation, intracellular pathogens and intestinal inflammation<sup>[26,27]</sup>. IL-12 exerts its biological function through the binding to its heterodimeric receptor formed by IL-12R- $\beta$ 1 and IL-12R- $\beta$ 2.  $\beta$ 2 receptor subunit plays a major role in IL-12 function, as it controls the Th1 cell lineage commitment. Moreover, IL-12R- $\beta$ 2 is overexpressed by T cells in inflamed mucosa<sup>[26]</sup> as well as in CD T-lamina propria lymphocytes (T-LPL)<sup>[28]</sup>. Data has shown that the inhibition of IL-12 resulted in reduced production of IFN- $\gamma$  and IL-21 in CD mucosal T cells cultures<sup>[23]</sup>, and T cell stimulation in T cell cultures from fetal gut explants, promoting Th1 immune response and causing mucosal degradation<sup>[29]</sup>. Beyond the role of IL-12 in T cells, a recent study has demonstrated its role in a distinct population, the innate lymphoid cells (ILCs), which are considerably encountered in inflamed tissue of intestinal wall of CD patients. IL-12 stimulates these cells to produce IFN- $\gamma$  indicating the role of ILCs in the pathogenesis of gut mucosal inflammation<sup>[30]</sup>.

Recent studies have demonstrated the crucial role of IL-23 in the regulation of Th1 cell responses and its potential role in the CD pathogenesis. IL-23 belongs to the IL-12 family, it is composed of one subunit of p40, that is shared with IL12, and one subunit of p19, which is unique<sup>[31]</sup>. IL-23 is produced by myeloid DCs or conventional DCs and macrophages in response to bacterial stimulation, endogenous signals or CD40L activation<sup>[32,33]</sup>. Depending on the various environmental signals, macrophages can obtain distinct functional phenotypes through undergoing different polarization<sup>[34]</sup>. Macrophage M1 phenotype is induced by microbial products or proinflammatory cytokines and is characterized by high production of IL-12 and IL-23 cytokines, in contrast to M2 phenotype which is mainly associated with Th2 immune responses and promotes tissue repair<sup>[35]</sup>. The binding of IL-23 on its

receptor, which is composed of IL-12R  $\beta$ 1 and IL-23R results in the specific induction of naïve CD4<sup>+</sup> T cells into Th17 cells, with a concomitant activation of numerous proinflammatory cytokines such as IL-17, IL-17F, TNF- $\alpha$  and IL-6<sup>[36]</sup>. Beyond CD4<sup>+</sup> T induction, IL-23 participates in the ILCs<sup>[37]</sup>, CD8<sup>+</sup> T<sup>[38]</sup>, natural killer (NK), NKT<sup>[39]</sup> and  $\gamma\delta$  T cells<sup>[40]</sup> activation. The presence of inflammation in the intestinal wall stems from the pathological Th1 immune response against the bacterial microbiota which are closely related to the IL-12 and IL-23 expression<sup>[41]</sup>. IL-23 expression is highly increased in ILCs in the inflamed intestine in CD patients, indicating the presence of IL-23-responsive ILCs in the human gut and promoting IL-17A and IFN- $\gamma$  production<sup>[37,42]</sup>. Moreover, studies have demonstrated the existence of single nucleotide polymorphisms (SNPs) in the IL-23R gene, which are highly protective in CD patients, suggesting that the blockade of IL-23 signaling could decrease the risk of CD development<sup>[43-45]</sup>.

The above data highlighted the determinant role of IL-12 and IL-23 in intestinal inflammation, since they are able to trigger signals in different cell populations and lead to the introduction of monoclonal antibodies as therapeutic agents for CD, targeting the common p40 subunit of IL-12 and IL-23.

## IL-12/IL-23 BLOCKADE/EMERGING BIOLOGICAL AGENTS

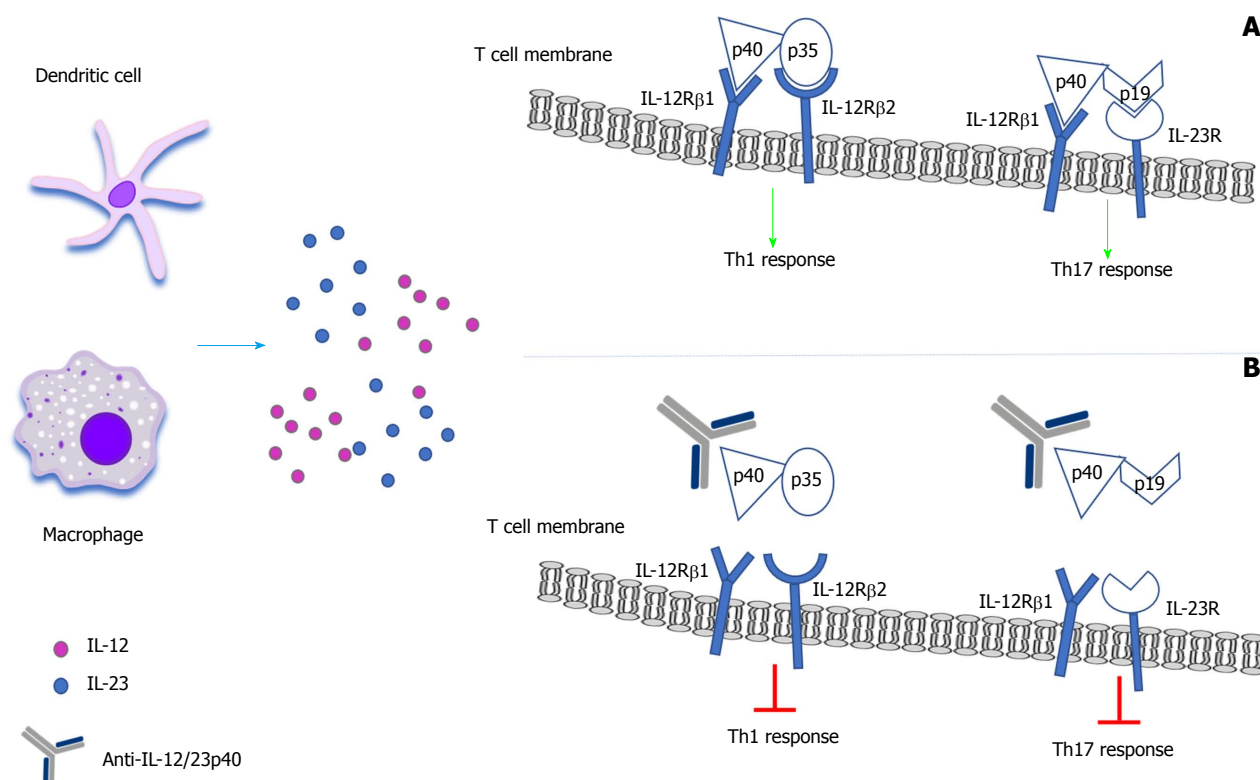
### Anti-IL-12/IL-23p40 antibodies

Ustekinumab is an IgG1 humanized monoclonal antibody directed against the common p40 subunit of the IL-12 and IL-23. It binds to the p40 subunit and impedes the interaction with the IL-12R $\beta$ 1 on the cell surface of NK, T cells, or antigen-presenting cells<sup>[46]</sup>. This process results in the blockade of IL-12 and IL-23 mediated downstream cell signaling, gene activation and cytokine production<sup>[46]</sup>. Ustekinumab binding to the IL-12 and IL-23, equally neutralizes IL-12 mediated responses, including the intracellular phosphorylation of STAT4, the expression of cell surface markers and the production of IFN- $\gamma$  and IL-23 mediated responses including the intracellular phosphorylation of STAT3 and IL-17A, IL-17F, and the production of IL-22<sup>[46]</sup> (Figure 1).

Briakinumab is an IgG1 monoclonal antibody with similar mechanism of action as ustekinumab, which was tested for the induction and maintenance of remission in patients with moderately to severely active CD. The results of a placebo-controlled phase 2b trial showed that although briakinumab presented a similar safety and tolerability profile to placebo in the induction and maintenance phases, it did not achieve the primary end point of clinical remission at week 6<sup>[47]</sup>.

### Anti-IL-23p19 antibodies

MEDI2070 (or AMG 139) is a humanized monoclonal



**Figure 1 Neutralization of the interleukin-12/interleukin-23 pathways associated with T-cell activation and differentiation.** A: In inflamed tissue, bacterial stimulation can lead to the activation of dendritic cells and macrophages. This process results in the activation of T cells and the secretion of inflammatory cytokines such as IL-12 and IL-23. The binding of IL-12 in its receptor, which is composed of IL-12R-β1 and IL-12R-β2 results in the preferential T cell differentiation into Th1 cells, promoting the Th1 cell response and secreting cytokines such as IFN-γ and TNF. The binding of IL-23 in its receptor, which is composed of IL-12R-β1 and IL-23R results in the preferential induction of T cells into Th17 cells, inducing Th17 cell response and secreting cytokines such as IL-17 and IL-22; B: The use of a monoclonal antibody against the common subunit of IL-12 and IL-23 (IL-12/23p40) that selectively targets both IL-23 and IL-12 cytokines, disrupts their mediated signaling pathway and cytokine cascade, through the prevention of these cytokines' interaction with their shared cell-surface receptor, IL-12R-β1. This process results in the inhibition of IL-12 and IL-23 signaling and further activation of Th1 and Th17 phenotypes. IL: Interleukin; IFN: Interferon; TNF: Tumor necrosis factor.

IgG2 antibody that selectively binds the p19 subunit, specifically blocking the binding of IL-23 to its receptor. In a phase 2a trial of patients with moderate to severe CD who had failed to anti-TNF treatment, the use of MEDI2070 showed induction of clinical response in CD patients compared to placebo<sup>[48]</sup>.

Risankizumab, a humanized monoclonal antibody targeting the p19 subunit of IL-23, resembles the mechanism of action of MEDI2070. The efficacy and safety of this antibody were assessed in a randomized, double-blind, placebo-controlled phase 2 study and the results showed that it was significantly better than placebo in inducing clinical remission<sup>[49]</sup>.

## USTEKINUMAB

Ustekinumab was approved in 2017 by the United States Food and Drug Administration (FDA) for the treatment of moderate to severe active CD in patients who have failed or were intolerant to therapy with corticosteroids or other immunomodulators but have never failed anti-TNF treatment, or in patients who have failed or were intolerant to therapy with one or more anti-TNF agents<sup>[50]</sup>. In parallel, in the same year, the use of

ustekinumab was approved by the European Medicines Agency (EMA) for adults with moderate to severe active CD with inadequate response, or loss of response, or intolerance to either conventional treatment, or anti-TNF agents, or with medical contraindications to such therapies<sup>[51]</sup>.

## CLINICAL EFFICACY OF USTEKINUMAB IN CD/ RANDOMIZED CLINICAL TRIALS

The clinical efficacy of ustekinumab in humans was first evaluated in immune-mediated diseases, such as psoriasis<sup>[52,53]</sup>, psoriatic arthritis<sup>[54-56]</sup> and multiple sclerosis<sup>[57]</sup>. The current use of ustekinumab in patients with CD has been assessed by multiple clinical trials (Tables 1 and 2).

### Phase II studies

The use of ustekinumab in the treatment of moderate to severe CD was first investigated in 2008 in a randomized, placebo-controlled, phase 2a induction trial<sup>[58]</sup>. The study comprised of two patient groups. Population 1 (the double-blind, cross-over phase IIa arm of the study) included 104 patients who had previously

**Table 1 Summary of randomized, placebo-controlled trials on ustekinumab in Crohn's disease**

| Study (reference)  | Publication year | Type of publication | Study design   | Study phase                                     |
|--|------------------|---------------------|--|---|
| Sandborn <i>et al</i> <sup>[58]</sup>                                | 2008             | Full paper          | Multicenter, double-blind, placebo-controlled, parallel cross-over | IIa   |
| Sandborn <i>et al</i> <sup>[59]</sup> (CERTIFI)                      | 2012             | Full paper          | Randomized, multicenter, double-blind, placebo-controlled          | IIb induction<br>IIb maintenance                |
| Feagan <i>et al</i> <sup>[60]</sup> (UNITI-1)                        | 2016             | Full paper          | Randomized, multicenter, double-blind, placebo-controlled          | III   |
| Feagan <i>et al</i> <sup>[60]</sup> (UNITI-2)                        | 2016             | Full paper          | Randomized, multicenter, double-blind, placebo-controlled          | III   |
| Feagan <i>et al</i> <sup>[60]</sup> (IM-UNITI)                       | 2016             | Full paper          | Randomized, multicenter, double-blind, placebo-controlled          | Maintenance phase of UNITI 1 and 2 responders   |
| Sandborn <i>et al</i> <sup>[63]</sup> (IM-UNITI long term extension) | 2017             | Abstract            | Randomized, multicenter, double-blind, placebo-controlled          | Maintenance phase of UNITI 1 and 2 responders   |
| Sands <i>et al</i> <sup>[62]</sup> (UNITI-IM)                        | 2018             | Abstract            | Randomized, multicenter, double-blind, placebo-controlled          | Maintenance phase of UNITI 1 and 2 responders   |
| Rutgeerts <i>et al</i> <sup>[61]</sup>                               | 2018             | Full paper          | Randomized, multicenter, double-blind, placebo-controlled          | Induction and maintenance of endoscopic healing |

received conventional therapy or anti-TNF regimens. The second group, population 2 - open-label arm, consisted of 27 non-responders (primary or secondary) to infliximab. The results showed that ustekinumab could induce clinical response in patients with moderate-to-severe active CD, especially in those who were previously treated with infliximab<sup>[58]</sup>. Regarding the development of serious adverse events, there was no difference in patients receiving ustekinumab compared to placebo<sup>[58]</sup>. The above results led to the conduct of a 36-wk, randomized, double-blind, placebo-controlled phase IIb trial (CERTIFI) on the role of ustekinumab in the induction and maintenance of remission in patients with moderate-to-severe CD who were resistant to anti-TNF treatment<sup>[59]</sup>. The study enlisted 526 patients in the induction arm and 145 responders in the maintenance phase. The results demonstrated that patients who were resistant to anti-TNF therapy showed an increased response rate to induction with ustekinumab compared to placebo, although remission rates were comparable<sup>[59]</sup>. However, ustekinumab induction responders showed significantly increased rates of response and remission during the maintenance phase<sup>[59]</sup>. No difference was reported in the incidence of adverse events between examined groups during the maintenance phase<sup>[59]</sup>. Basal-cell carcinoma developed in 1 patient receiving ustekinumab.

### Phase III studies

Phase III, multicentre, double-blind, placebo-controlled, trials for the evaluation of ustekinumab in patients with moderate to severe CD have been recently completed. The first trial (UNITI-1) included 741 patients who were primary or secondary non-responders to anti-TNF treatment or had severe side effects<sup>[60]</sup>. In the second trial (UNITI-2) 628 patients who had failed the conventional therapy or had experienced severe side effects were enrolled<sup>[60]</sup>. The results showed that intravenous ustekinumab induced clinical response and

remission in patients from both trials (UNITI 1-2)<sup>[60]</sup>. No difference in adverse and serious adverse events was reported between the groups. Moreover, there was no report of death, malignancy, opportunistic infections or tuberculosis in ustekinumab treated patients<sup>[60]</sup>. The 397 patients who completed the induction trials (UNITI 1 and 2) and were responders to ustekinumab, were enrolled in the IM-UNITI trial<sup>[60]</sup>. Primary endpoint for this trial was the maintenance of remission at week 44 and the results showed that treatment with ustekinumab was more effective than placebo for maintaining remission<sup>[60]</sup>. Between the placebo and the ustekinumab groups, the rates of adverse events development and severity were similar<sup>[60]</sup>.

### Effect of ustekinumab in endoscopic activity

A sub-study of the UNITI trial enrolled 334 patients with moderate to severe CD and assessed the clinical effect of ustekinumab in the simplified endoscopic activity score for CD (SES-CD) and the efficacy of maintenance therapy<sup>[61]</sup>. Patients treated with ustekinumab had higher reduction in SES-CD compared to placebo during the induction phase<sup>[61]</sup>. The results were similar in patients from different induction trials (UNITI 1 or 2) and in those receiving different ustekinumab doses. Greater reduction in the SES-CD at week 44 was also observed in the ustekinumab group compared to placebo<sup>[61]</sup>.

### Dose adjustment effect of ustekinumab in patients with loss of response or in delayed responders

Another sub-study of the UNITI-IM maintenance programme addressed important points of clinical application of ustekinumab. This trial evaluated the clinical effect of dose adjustment of ustekinumab in patients who (1) entered the maintenance trial in response and subsequently lost response (LOR) (2) were non-responders to intravenous ustekinumab during induction phase<sup>[62]</sup>. The results showed that in patients



**Table 2** Characteristics of randomized, placebo-controlled trials evaluating the efficacy of ustekinumab in Crohn's disease

| Study (reference)  | Patients   | Endpoints   | Intervention parameters   | Outcomes                 |
|--|--|---|---|--------------------------|
| Sandborn <i>et al</i> <sup>[58]</sup>  | 25   | Clinical response at week                         | 90 mg SC week 0-3→Placebo SC week 8-11                              | Ustekinumab:             |
|  | 26   | 4/week 8  | Placebo SC week 0-3→90mg SC week 8-11                               | 53%/49%                  |
|  | 26   |   | 4.5 mg/Kg IV week 0→Placebo IV week 8                               | Placebo:                 |
|  | 27   |   | Placebo IV week 0→4.5 mg/Kg IV week 8                               | 30%/40%                  |
| Sandborn <i>et al</i> <sup>[59]</sup> (CERTIFI)  | 27 (primary or secondary non-responders to infliximab) | Clinical response at week 8                       | 90 mg SC  | 43%                      |
|  |  |   | 4.5 mg/kg IV  | 54%                      |
|  | 131  | Clinical response at week 6                       | 1 mg/kg IV  | 36.60%                   |
|  | 132  |   | 3 mg/kg IV  | 34.10%                   |
| Induction  | 131  |   | 6 mg/kg IV  | 39.70%                   |
|  | 132  |   | Placebo IV  | 23.50%                   |
|  | 145 Responders   | Clinical response at week 22                      | 90 mg SC  | 69.40%                   |
|  | 72 Ustekinumab   |   | Placebo SC  | 42.50%                   |
| Sandborn <i>et al</i> <sup>[59]</sup> (CERTIFI) Maintenance  | 73 Placebo   | Clinical remission at week 22                     | 90 mg SC  | 41.70%                   |
|  |  |   | Placebo SC  | 27.40%                   |
|  |  |   | 130 mg IV   | 34.30%                   |
|  |  |   | 6 mg/kg IV  | 33.70%                   |
| Feagan <i>et al</i> <sup>[60]</sup> UNITI-1 induction  | 245  | Clinical response at week 6                       | Placebo IV  | 21.50%                   |
|  | 249  |   | 130 mg IV   | 51.70%                   |
|  | 247  |   | 6 mg/kg IV  | 55.50%                   |
|  |  |   | Placebo IV  | 28.70%                   |
| Feagan <i>et al</i> <sup>[60]</sup> UNITI-2 induction  | 209  | Clinical response at week 6                       | 130 mg IV   | 51.70%                   |
|  | 209  |   | 6 mg/kg IV  | 55.50%                   |
|  | 210  |   | Placebo IV  | 28.70%                   |
|  |  |   |   |                          |
| Feagan <i>et al</i> <sup>[60]</sup> IM- UNITI maintenance  | 132  | Clinical remission at week 44                     | 90 mg SC every 8 wk   | 53.10%                   |
|  | 132  |   | 90 mg SC every 12 wk  | 48.80%                   |
|  | 133  |   | Placebo SC  | 35.90%                   |
|  |  |   |   |                          |
| Sandborn <i>et al</i> <sup>[63]</sup> (IM- UNITI long term extension)  | 1281   | Clinical remission at week 92                     | 90 mg SC every 8 wk   | 74.40%                   |
|  |  |   | 90 mg SC every 12 wk  | 72.60%                   |
|  |  |   | Subjects with prior dose adjustment                                 | 53.50%                   |
|  |  |   | All ustekinumab treated   | 67.50%                   |
| Sands <i>et al</i> <sup>[62]</sup> (IM-UNITI patients with dose adjustment following loss of response)           | 51   | Clinical response [CR-100] <sup>1</sup>           | Placebo to 90 mg SC ustekinumab every 8 wk                          | 71%                      |
|  | 29   |   | Ustekinumab 90 mg SC every 12 wk to ustekinumab 90 mg SC every 8 wk | 55%                      |
|  | 28   |   | No dose adjustment  | 46%                      |
|  |  |   | Additional dose of 90 mg SC   | 50.50%                   |
| Sands <i>et al</i> <sup>[62]</sup> (IM-UNITI non-responders during induction phase having an additional SC dose) | 467  | Clinical response 8 wk after one additional dose  |   |                          |
|  |  | Clinical remission 8 wk after one additional dose |   | 28.90%                   |
|  |  |   |   |                          |
|  |  |   |   |                          |
| Rutgeerts <i>et al</i> <sup>[61]</sup> Induction week 8  | 155  | SES-CD Change from baseline, mean (SD)            | 130 mg IV/6 mg/kg   | -2.8 (8.10) <sup>a</sup> |
|  | 97   |   | Placebo IV  | -0.7 (4.97)              |
|  | 47   | SES-CD Change from baseline, mean (SD)            | 90mg SC every 12 wk   | -1.5 (4.22)              |
|  | 74   |   | 90mg SC every 8 wk  | -3.8 (6.02)              |
| Rutgeerts <i>et al</i> <sup>[61]</sup> Maintenance week 44   | 51   |   | Placebo SC  | -2.0 (5.35)              |
|  |  |   |   |                          |

<sup>1</sup>CR-100, ≥ 100-point decrease in Crohn's Disease Activity Index; <sup>a</sup>P < 0.05. SC: Subcutaneous; IV: Intravenous; SES-CD: Simplified endoscopic activity score for Crohn's disease; SD: Standard deviation.

with LOR, the dose adjustment of ustekinumab (12-wk interval to 8-wk interval) provided clinical benefits compared to patients who remained to the 8-wk interval. Moreover, patients who were initial non-responders to induction treatment benefited from continued treatment (at least 1 additional subcutaneous dose) following the initial intravenous dose (rescue therapy - late responders)<sup>[62]</sup>.

### Long-term efficacy and safety of ustekinumab

The long-term efficacy and safety of ustekinumab were evaluated in an ongoing IM-UNITI study with a duration of approximately 5 years<sup>[63]</sup>. The preliminary results through week 96 showed that the clinical response and remission were maintained in patients who were under treatment with subcutaneous ustekinumab. There was no difference in adverse events and infection rates

between patients treated with ustekinumab or placebo from week 44 through week 96<sup>[63]</sup>.

## DISCUSSION

The introduction of monoclonal antibodies in the last decades has changed the therapeutic strategy of CD. The biological factors that have been approved to date for the management of CD include anti-TNF agents (adalimumab, certolizumab and infliximab), anti-integrins (natalizumab and vedolizumab) and the anti-IL-12/IL-23p40 agent (ustekinumab). The clinical benefits of monoclonal antibodies are the efficacy and safety during the induction and maintenance of clinical response as well as a decreased risk of hospitalization and surgery. Anti-TNF agents are currently positioned as first line biologic treatment for the management of

moderate to severe CD and have been proven to be effective for both induction and maintenance of CD patients<sup>[64]</sup>. However, these agents do not fully cover the needs of all patients as there is significant percentage who was not respond to treatment, or even if they achieved an initial short-term response, they could undergo secondary failure to anti-TNF agents or develop unacceptable adverse events, which lead to treatment discontinuation. Currently, for patients with primary failure to anti-TNF treatment, the use of a second TNF agonist is not indicated and a switch to another agent with a different mechanism of action is suggested<sup>[65,66]</sup>. The anti- $\alpha 4\beta 7$  integrin antibody was approved for induction and maintenance of CD patients, but due to its fairly slow-action, it is considered better for the maintenance phase.

Clinical evidence suggests that ustekinumab may be preferred over the anti-integrin treatment given its rapid onset of action<sup>[60]</sup>. In particular, the clinical benefits of ustekinumab over vedolizumab in inducing clinical response and remission have been shown in patients who were non-responders or were intolerant to anti-TNF treatment, since ustekinumab treated patients responded as early as week 3<sup>[60]</sup> compared to patients treated with vedolizumab who responded at week 10<sup>[67]</sup>. The results of the study IM-UNITI LTE have shown that the rapid onset of ustekinumab action is accompanied by a long duration of action, as 75% of patients in remission at year 1, were still in remission at year 2, indicating one more advantage over the anti-TNF or anti-integrins agents<sup>[63]</sup>.

The route of ustekinumab administration could be considered as an important benefit over the other treatment options. During induction phase, only one intravenous dose of ustekinumab is required for the development of clinical response and during maintenance phase, a single subcutaneous dose is able to induce clinical response up to 44 wk in one-third of the patients<sup>[60]</sup>. These results highlight the usefulness of ustekinumab as a more convenient option with its potential for a home-based therapy. Moreover, a sub-study of the IM-UNITI programme has shown that ustekinumab dose adjustment can provide additional clinical advantage in patients with loss of response. The results demonstrated that initial non-responders to the induction therapy could benefit more from continued treatment with at least one additional dose<sup>[62]</sup>.

The study by Fegan *et al.*<sup>[60]</sup>, has shown that the rapid onset of clinical efficacy was accompanied by a reduction in CRP and fecal calprotectin levels which persisted during the maintenance phase up to week 44. The improvement in CRP and fecal calprotectin levels following ustekinumab treatment suggests that decrease of inflammation occurred along with the clinical improvement<sup>[60]</sup>. In parallel, recent data has demonstrated the ability of ustekinumab to induce endoscopic healing during the induction phase (at week

8) in patients with moderate to severe CD<sup>[61]</sup>.

Beyond the obvious benefits of ustekinumab in the management of moderate to severe CD, patient safety is an important factor of determining the risk/benefit ratio of each treatment option. Although the data from studies evaluating the long-term safety of ustekinumab in patients with moderate to severe psoriasis and multiple sclerosis suggests an increased risk of serious infections, in CD patients the drug seems to have a rather favorable safety profile<sup>[58-60,63]</sup>, which is very important considering the role of IL-12/IL-23 in maintaining immune homeostasis<sup>[68-71]</sup>.

Taking into consideration the above advantages, we can speculate the use of ustekinumab as a first-line treatment or its use in conjunction with or before other biological agents, following corticosteroid failure. Ustekinumab may be the ideal option for frail patients, considering its safety profile and the mode of administration. In addition, CD patients with other immune mediated diseases such as previous history of multiple sclerosis and psoriasis or patients with TNF agonist induced psoriasis represent promising candidates for ustekinumab treatment considering its systemic anti-inflammatory action<sup>[52-56,72,73]</sup>. On the other hand, the use of ustekinumab may not be favored in patients with perianal fistulizing CD in whom the use of infliximab is proposed<sup>[74]</sup>. Furthermore, there is limited evidence concerning the use of ustekinumab in pregnancy and breast feeding. Studies in animals have shown no developmental toxicity due to ustekinumab exposure<sup>[75]</sup>. In humans, there are two case reports of abortions in ustekinumab-exposed pregnancies<sup>[76,77]</sup> and two successful pregnancies after prolonged ustekinumab treatment<sup>[78,79]</sup>.

## CONCLUSION

Ustekinumab has exhibited considerable potential in the management of intestinal inflammation by downregulating the immune system through its binding in the common subunit of IL-12 and IL-23 and by blocking their action. In light of current evidence, ustekinumab is considered to be an effective drug with a favorable safety profile for the management of patients with moderate-to-severe active CD. Its use appears to be very promising in patients with an inadequate response, loss of response, intolerance or contraindications to treatment with traditional anti-TNF agents. Moreover, ustekinumab could be considered as first line biological treatment in patients who failed conventional therapy, although the high treatment cost poses severe limitations to this alternative. Large scale-multicentre trials with long term follow up and high-quality evidence are required to further explore the spot that this novel agent should hold in the CD treatment algorithms, and its role in specific disease phenotypes (such as fistulizing disease, early-onset

CD or postoperative setting). Lastly, conduct of head-to-head comparison studies of ustekinumab with other biological agents, evaluation of drug to drug interactions and pharmacoeconomic (cost effective) analysis of ustekinumab are the next steps towards thoroughly delineating the place of ustekinumab in clinical practice.

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## Role of microRNAs in alcohol-induced liver disorders and non-alcoholic fatty liver disease

Jorge-Luis Torres, Ignacio Novo-Veleiro, Laura Manzanedo, Lucía Alvela-Suárez, Ronald Macías, Francisco-Javier Laso, Miguel Marcos

Jorge-Luis Torres, Laura Manzanedo, Ronald Macías, Francisco-Javier Laso, Miguel Marcos, Department of Internal Medicine, University Hospital of Salamanca, Institute of Biomedical Research of Salamanca-IBSAL, Salamanca 37007, Spain

Francisco-Javier Laso, Miguel Marcos, Department of Medicine, Faculty of Medicine, University of Salamanca, Salamanca 37007, Spain

Ignacio Novo-Veleiro, Department of Internal Medicine, University Hospital of Santiago de Compostela, A Coruña 15706, Spain

Lucía Alvela-Suárez, Department of Internal Medicine, HM Rosaleda Hospital, Santiago de Compostela, A Coruña 15701, Spain

Jorge-Luis Torres, Ignacio Novo-Veleiro, Francisco-Javier Laso, Miguel Marcos, Spanish Working Group on Alcohol and Alcoholism, Spanish Society of Internal Medicine, Madrid 28016, Spain

ORCID number: Jorge-Luis Torres (0000-0001-6853-9115); Ignacio Novo-Veleiro (0000-0003-0948-2440); Laura Manzanedo (0000-0001-8394-5218); Lucía Alvela-Suárez (0000-0001-6106-2174); Ronald Macías (0000-0001-8734-7872); Francisco-Javier Laso (0000-0003-0945-5186); Miguel Marcos (0000-0003-1269-4487).

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**Correspondence to:** Miguel Marcos, MD, PhD, Associate Professor, Staff Physician, Department of Internal Medicine, University Hospital of Salamanca, Paseo de San Vicente 182, Salamanca 37007, Spain. [mmarcos@usal.es](mailto:mmarcos@usal.es)  
**Telephone:** +34-923-291100-55437  
**Fax:** +34-923-294739

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### Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that regulate multiple physiological and pathological functions through the modulation of gene expression at the post-transcriptional level. Accumulating evidence has established a role for miRNAs in the development and pathogenesis of liver disease. Specifically, a large number of studies have assessed the role of miRNAs

in alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD), two diseases that share common underlying mechanisms and pathological characteristics. The purpose of the current review is to summarize and update the body of literature investigating the role of miRNAs in liver disease. In addition, the potential use of miRNAs as biomarkers and/or therapeutic targets is discussed. Among all miRNAs analyzed, miR-34a, miR-122 and miR-155 are most involved in the pathogenesis of NAFLD. Of note, these three miRNAs have also been implicated in ALD, reinforcing a common disease mechanism between these two entities and the pleiotropic effects of specific miRNAs. Currently, no single miRNA or panel of miRNAs has been identified for the detection of, or staging of ALD or NAFLD. While promising results have been shown in murine models, no therapeutic based-miRNA agents have been developed for use in humans with liver disease.

**Key words:** Alcohol use disorder; Alcoholic liver disease; Non-alcoholic fatty liver disease; Steatosis; Obesity; miRNA; Biomarkers

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**Core tip:** MicroRNAs (miRNAs) are small RNAs that regulate gene expression at a post-transcriptional level. Altered miRNA expression has been found in a variety of liver diseases, including non-alcoholic fatty liver disease and alcoholic liver disease. A group of miRNAs (miR-155, miR-122 and miR-34a) contributes to the pathogenesis of these two diseases and these miRNAs have potential use as biomarkers or therapeutic targets. Several technical limitations and a lack of clinical studies, however, preclude their clinical use.

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## INTRODUCTION

MicroRNAs (miRNAs), small non-coding RNAs, can modulate gene expression at the post-transcriptional level by targeting messenger RNAs and inhibiting their translation or promoting their degradation<sup>[1,2]</sup>. Since the discovery of the first miRNA in 1993, lin-4<sup>[3]</sup>, more than 2000 miRNAs have been described in humans and they are believed to regulate up to 60% of protein-coding genes in the human genome<sup>[4]</sup>.

Human miRNAs are involved in virtually all physiological and pathological processes, including cell differentiation and proliferation, signal transduction, inflammation and immune response, metabolism, viral-

host interaction, and oncogenesis<sup>[1,2]</sup>. The expression of a wide variety of miRNAs is potentially regulated by many factors, such as alcohol, but also diet, cigarette smoking and other drugs<sup>[5]</sup>. Therefore, it is not surprising that miRNAs have been increasingly recognized as key actors in the pathogenesis of a variety of diseases and as potential biomarkers for diagnosis or therapeutic targets<sup>[2]</sup>. The role of miRNAs in liver inflammation, fibrosis and cirrhosis has been widely described in the last twenty years<sup>[6-8]</sup>. The current paper reviews the existing literature pertaining to miRNA alteration, function, and the potential clinical application of miRNAs in alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD). While ALD and NAFLD differ in some aspects, they also share common features, including underlying mechanisms and clinical and histopathological characteristics<sup>[9]</sup>. Given the rapid expansion of research in miRNAs in recent years, an updated review on the topic will first be presented, followed by a summary of miRNA alterations that are common to both ALD and NAFLD.

## ROLE OF MIRNAS IN ALD

### *Pathogenic role of miRNAs in ALD*

The development of the different forms of ALD (steatosis, alcoholic hepatitis and cirrhosis) requires prolonged and heavy alcohol consumption along with susceptibility to the disease. Pathophysiological mechanisms of ALD are based both on the direct toxic effect of alcohol and also on ethanol-induced alterations in the inflammatory response<sup>[10]</sup>. A variety of enzymes, such as alcohol dehydrogenase (ADH) and the cytochrome P450 2E1 (CYP2E1), contribute to alcohol metabolism<sup>[11]</sup>, leading to oxygen free radicals, nitric oxide and acetaldehyde, which ultimately can cause cellular damage and liver inflammation<sup>[12]</sup>. In addition, the toxic effect of acetaldehyde increases intestinal permeability to bacterial lipopolysaccharide (LPS)<sup>[13]</sup>, which binds to toll-like receptors 4 (TLR4) and activates Kupffer and stellate cells through pro-inflammatory cytokines, such as tumour necrosis factor (TNF)- $\alpha$ , production<sup>[14]</sup>. This inflammatory signal is transmitted via the nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway, ultimately leading to liver damage<sup>[14]</sup>.

While most immune mechanisms involved in ALD development are related to the TLR4-NF- $\kappa$ B pathway, the activation of TLR4 also triggers the transmission of pro-inflammatory stimuli through other signaling pathways, such as mitogen-activated protein kinases (MAPK) or TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF)<sup>[14]</sup>. miRNAs can regulate this complex interplay between inflammatory signals via the regulation of cytokines and other components of the pathways<sup>[15]</sup>. Oxidative stress and free oxygen radicals generation involved in ALD development are also regulated by miRNAs through different pathways like Kelch-like ECH-associated protein 1 Kelch-like ECH-associated protein 1 (Keap1) / Nuclear factor-erythroid-2-related factor 2 (Nrf2) pathway<sup>[16-20]</sup>. In addition to this, miRNAs have also



**Table 1** MicroRNA targets involved in alcoholic liver disease pathogenesis

| miRNA                                 | Source of sample                        | miRNA target   |
|---------------------------------------|---|--|
| let-7 <sup>[27]</sup>                 | Animal models                           | Lin28, HMGA2   |
| miR-19b <sup>[28]</sup>               | Human HSCs                              |  |
|                                       | Animal models                           | TGFβRII, Col1α2, MeCP2                                 |
|                                       | Human HSCs                              |  |
| miR-21 <sup>[36,37]</sup>             | Animal models                           | FASLG, DR5, Creb12                                     |
| miR-26a <sup>[35]</sup>               | Animal models                           | DUSP4, DUSP5   |
| miR-27a <sup>[44,52]</sup>            | Animal models                           | Sprouty2, CD206  |
|                                       | HMC                                     |  |
|                                       | Humans (plasma)                         |  |
| miR-34a <sup>[29,43]</sup>            | Animal models                           | SIRT1, CASP2   |
|                                       | Human HSCs                              |  |
|                                       | NHH                                     |  |
|                                       | HiBECs                                  |  |
|                                       | Humans (liver biopsy)                   |  |
| miR-103 and miR-107 <sup>[53]</sup>   | Humans (liver biopsy)                   | Caveolin-1   |
| miR-122 <sup>[32,124,125]</sup>       | Animal models                           | P4HA1, HO-1, Cyclin G1, Bcl-w, HIF-1α                  |
| miR-155 <sup>[38,39,97,126,127]</sup> | Animal models                           | TNFα, SHIP1, SOCS1, IRAKM, C/EBPβ                      |
| miR-181b-3p <sup>[40]</sup>           | Animal models                           | Importin α1  |
| miR-182 <sup>[30]</sup>               | Animal models                           | SLC1A1, Cofilin 1, CCL20, CXCL1, IL-8, Cyclin D1, IL-6 |
|                                       | Humans (serum samples and liver biopsy) |  |
| miR-199 <sup>[128]</sup>              | Animal models                           | ET-1, ET-BR  |
| miR-200a <sup>[31]</sup>              | Animal models                           | ZEB-2  |
| miR-212 <sup>[46]</sup>               | Caco-2 cells                            | ZO-1   |
|                                       | Humans (colon biopsy)                   |  |
| miR-214 <sup>[24,34]</sup>            | Animal models                           | POR, GSR, CYP2E1                                       |
|                                       | HHCs                                    |  |
| miR-217 <sup>[41]</sup>               | Animal models                           | SIRT-1   |
| miR-223 <sup>[45]</sup>               | Animal models                           | p47 <sup>phox</sup> , IL-6                             |
|                                       | Humans (serum)                          |  |
| miR-291b <sup>[42]</sup>              | Animal models                           | Tollip   |
|                                       | HPBMs                                   |  |
| miR-378 <sup>[59]</sup>               | Animal models                           | Gli-3  |
| miR-497 <sup>[25]</sup>               | Animal models                           | Btg2, Yy1  |

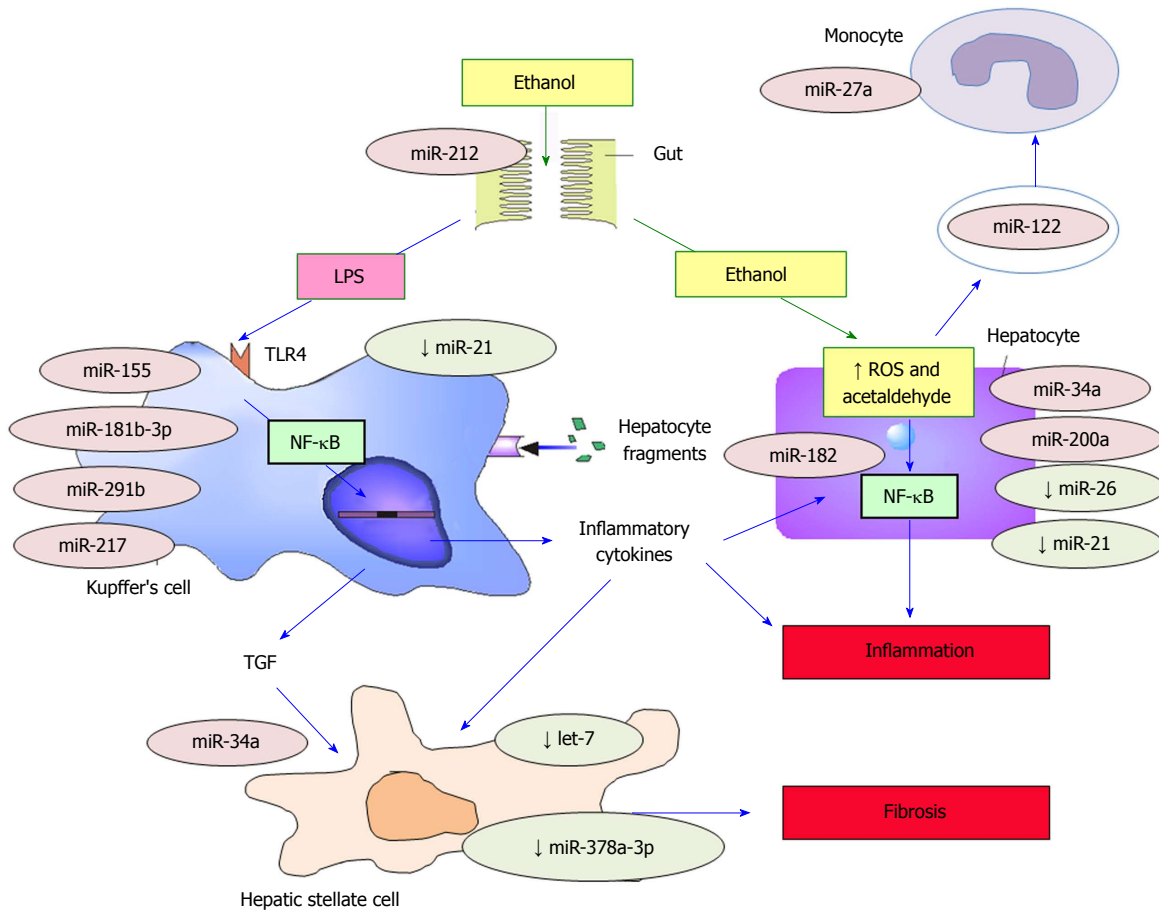
HSCs: Hepatic stellate cells; HMGA2: High mobility group AT-hook 2; TGFβRII: Transforming growth factor β receptor II; Col1α2: Collagen type I α 2 chain; MeCP2: Methyl-CpG binding protein 2; FASLG: Fas ligand; DR5: Death receptor 5; Creb12: cAMP responsive element binding protein like 2; DUSP: Dual specificity phosphatase; HMC: Human Monocyte Cells; NHH: Normal Human Hepatocytes; HiBECs: Human intrahepatic Biliary Epithelial Cells; SIRT1: sirtuin 1; CASP2: caspase 2; P4HA1: prolyl 4-hydroxylase subunit α 1; HO-1: heme oxygenase-1; BCL-W: Bcl-2-like protein 2; HIF-1α: Hypoxia inducible factor 1 α; TNFα: Tumor necrosis factor α; SHIP1: Src homology 2 domain-containing inositol phosphatase 1; SOCS1: Suppressor of cytokine signaling 1; IRAKM: Interleukin 1 receptor associated kinase 3; C/EBPβ: CCAAT/enhancer binding protein β; SLC1A1: Solute carrier family 1 member 1; CCL20: C-C motif chemokine ligand 20; CXCL1: C-X-C motif chemokine ligand 1; IL: Interleukin; ET-1: Endothelin-1; ET-BR: Endothelin-B receptor; ZEB-2: Zinc finger E-box binding homeobox 2; ZO-1: Zonula occludens 1; HHCs: Human Hepatoma Cells; POR: Cytochrome P450 oxidoreductase; GSR: Glutathione reductase; CYP2E1: Cytochrome P450 2E1; p47<sup>phox</sup>: Neutrophil cytosolic factor 1-like; HPBMs: Human Peripheral Blood Monocytes; Tollip: Toll interacting protein; Gli3: GLI Family Zinc Finger 3; Btg2: B-cell translocation gene 2; YY1: Yin yang 1; miRNA: MicroRNA.

been shown to exert an important modulatory function on macrophage activation and differentiation<sup>[21,22]</sup>. Moreover, recent studies have shown even broader effects of miRNAs in ALD development, including a role in intercellular communication, in secretion in exosomes<sup>[23]</sup>, in the expression of enzymes directly linked to alcohol metabolism (*e.g.*, regulation of CYP2E1 by miR-214<sup>[24]</sup>) and in the modulation of pro-inflammatory pathways such as the B-cell translocation gene 2/Yin-yang 1 (BTG2/YY1) signaling pathway by miR-497<sup>[25]</sup>. Finally, alcohol consumption, with or without concurrent ALD, has also been linked to altered expression of several miRNAs<sup>[5,26]</sup>.

Numerous studies, therefore, have addressed the relationship between ALD development and miRNAs. While animal models have been used in the majority of these studies, there is an increasing number of studies in human cells, tissues and serum, confirming the key

role of miRNAs in ALD<sup>[27-30]</sup>. A summary of all available studies is shown in Table 1. In addition, a summary of the regulatory actions of miRNAs in the inflammatory response according to the different cell types involved, is displayed in Figure 1.

**Hepatocytes:** Some miRNAs (*e.g.*, miR-34a and miR-200a) are responsible for the induction of hepatocytic apoptosis during ALD development<sup>[29,31]</sup>. In addition, secretion of miRNAs in exosomes (*e.g.*, miR-122) can cause an increase in inflammatory response by targeting monocyte/macrophage cells<sup>[32]</sup>, ultimately leading to hepatocytic injury. MiRNAs action and pleiotropic effects could be different depending on the cell in which they act; thus, miR-122 could have a protective role inside the hepatocyte during alcohol-induced liver damage<sup>[33]</sup>. Increase in oxidative stress



**Figure 1** MicroRNAs involved in the pathogenesis of alcoholic liver disease. miRNAs preceded by a ↓ symbol are decreased in ALD or inhibit the development of ALD. The remainder of miRNAs promotes the development of ALD. TLR4: Toll-like receptor 4; TGF: Transforming growth factor; ALD: Alcoholic liver disease; ROS: Reactive oxygen species; NF-κB: Nuclear factor-κB. Figure adapted from Laso *et al*<sup>[10]</sup>.

and alterations of enzymatic function in hepatocytes are also regulated by miRNAs<sup>[24,34]</sup>. Conversely, miRNAs may also have a protective role in ALD. For example miR-26a can increase autophagy<sup>[35]</sup> and miR-21 can inhibit alcohol-induced apoptosis<sup>[36,37]</sup>.

**Kupffer cells (KCs):** miR-155, which is increased by chronic alcohol consumption through NF-κB induction, has been shown to be the main regulator of KC activation and function<sup>[38]</sup>. miR-155 inhibits the expression of multiple TLR4/NF-κB inhibitory regulators such as Src homology 2 domain-containing inositol phosphatase 1 (SHIP1) and Suppressor of cytokine signaling 1 (SOCS1)<sup>[38,39]</sup> leading to an increase in KC response to LPS and ultimately the development of liver fibrosis<sup>[39]</sup>. The Keap1/Nrf2 pathway could also be involved in miR-155 role in ALD development and KCs regulation<sup>[17]</sup>. Other miRNAs, such as miR-181b-3p, are also linked to increased LPS-sensitivity through the TLR4-NF-κB pathway<sup>[40]</sup>. In addition, miRNAs have been shown to regulate Sirtuin-1-Lipin-1, an inflammatory response mediator, leading to the down-regulation of the NF-κB pathway *via* de-acetylation. Alcohol consumption increases miR-217 expression, which in turn down-regulates sirtuin-1-Lipin-1<sup>[41]</sup>, consequently leading to

more hepatic inflammation<sup>[41]</sup>. Toll Interacting Protein (Tollip), another down-regulator of the TLR4-NF-κB pathway, is inhibited by miR-291b<sup>[42]</sup>.

**Hepatic stellate cells (HSCs):** HSCs, responsible for the development of liver fibrosis, are regulated by several miRNAs, including let-7. The downregulation of let-7 by LPS and alcohol use causes an increase in HSCs activation<sup>[27]</sup>. In addition, chronic alcohol consumption has been linked to an overexpression of miR-34a, which increases the expression of proteins such as transforming growth factor-β1 (TGF-β1), leading to a higher survival of HSCs through apoptosis inhibition<sup>[43]</sup>.

**Other cell types:** In addition to the cell types described above, other cells involved in ALD development, such as circulating monocytes (by miR-27a<sup>[44]</sup>), and circulating neutrophils, (by miR-223<sup>[45]</sup>) are regulated by miRNAs. In addition, miR-212 has been shown to increase permeability to LPS by altering cells of the intestinal mucosa<sup>[46]</sup>.

Due to the role of miRNAs in ALD and the modulatory effects of alcohol consumption on miRNA expression, it is plausible to hypothesize that genetic variations in certain miRNAs may lead to altered miRNA function and

an increased risk of liver damage. Consequently, we and others have analyzed the relationship of alcohol-related diseases and polymorphisms within miRNA genes or miRNA targets<sup>[47,48]</sup>. Interestingly, the miR-146a C>G rs2910164 variant is linked to a susceptibility to alcohol use disorder<sup>[47]</sup> and the pre-miR-27a A>G rs895819 polymorphism is linked to a higher alcohol intake<sup>[49]</sup>, suggesting a potential relationship between these genetic variants and alcohol-related diseases. The lack of replication studies precludes any conclusions regarding these SNPs, and to date, only rs738409 polymorphism within the *PNPLA3* gene is clearly linked to a higher susceptibility to ALD<sup>[50]</sup>.

#### **miRNAs as a target for diagnosis and treatment of ALD**

The clinical use of miRNAs as a diagnostic tool or therapeutic agent in ALD has not been well studied<sup>[51]</sup>. However, over the last years, an increasing number of miRNAs have been proposed as potential biomarkers of ALD. The following is a review of the most promising results.

**miR-192 and miR-30a:** It has been shown that serum levels of miR-192 and miR-30a are significantly correlated with the diagnosis of alcoholic hepatitis. Therefore, these miRNAs may be useful in the diagnosis, staging, and monitoring of patients with this specific form of ALD<sup>[23]</sup>.

**miR-27a:** miR-27a has been linked to monocyte differentiation and is increased in extracellular plasmatic vesicles of patients with alcoholic hepatitis, making it a potentially useful diagnostic tool<sup>[52]</sup>.

**miR-182:** An elevated level of miR-182 has been linked to greater disease severity and liver injury in alcoholic hepatitis. The correlation between miR-182 and disease severity, however, has only been shown in liver biopsies, limiting its application as a diagnostic tool<sup>[30]</sup>.

**miR-103 and miR-107:** A prior study found that miR-103 and miR-107 were increased in liver from patients with ALD and with NAFLD, but not in healthy livers or in subjects with viral hepatitis<sup>[53]</sup>.

**miR-155 and miR-122:** Increased blood levels of miR-155<sup>[32,54]</sup> and miR-122<sup>[55]</sup> have been found in healthy individuals after binge drinking and in a murine model of liver damage. While these miRNAs could be potential biomarkers of alcohol intake or alcohol liver damage, they are increased in several types of liver disease and therefore are unlikely to be specific to ALD<sup>[54]</sup>.

#### **Therapeutic application of miRNAs in ALD**

There are no studies to date supporting a therapeutic role for miRNAs in ALD. Available data, however, suggest a potential role for the inhibition or activation of some

miRNAs in the treatment of liver disease. A recent study found that treatment with hyaluronic acid normalized miR-181b-3p and Importin  $\alpha$ 5 levels in ethanol-fed mice, protecting them from ethanol-induced liver and intestinal damage<sup>[40]</sup>. In addition, hyaluronic acid normalized the miR-291b/Tollip pathway, leading to a lower sensitization of monocytes/macrophages to ethanol-induced activation *via* TLR4<sup>[42]</sup>. While both studies were performed in animal models, taken together they suggest a potential role for hyaluronic acid as a therapeutic regulator of the KC response to ethanol *via* miRNA modulation.

The role of miR-155 in KC and miR-122 in hepatocytes suggest that these miRNAs may serve as potential targets for treatment of ALD. Miravirsin, an miR-122 inhibitor, has shown promising results in chronic hepatitis C treatment<sup>[56,57]</sup>, suggesting its potential usefulness in ALD. A recent study showed that the restoration of miR-122 in hepatocytes could have a protective role against ALD development<sup>[33]</sup>. These apparently contradictory results could reflect the ability of miRNAs to develop different actions in different cells and also its relevance in inter-cellular communications<sup>[32]</sup>. In this sense, the therapeutic action of Miravirsin over viral replication could be explained by the interruption of these communications<sup>[57]</sup>. In addition, other potential therapeutic miRNAs currently under development for other diseases, such as cardiac fibrosis and remodeling or vascular disease<sup>[58]</sup>, could serve as potential targets for ALD. There is indirect data that inhibition of miR-155, may lead to decreased sensitivity of KC to LPS-mediated activation<sup>[39]</sup>.

In addition to the inhibition of detrimental miRNAs, stimulation of protective miRNAs could also serve as a potential therapeutic target. For example, miR-21, which aids hepatocyte regeneration<sup>[36]</sup>; miR26a, which protects hepatocytes from fibrosis development<sup>[35]</sup>; miR-223, which inhibits neutrophil activation and liver infiltration<sup>[45]</sup>; and miR-378, which exerts a stop-signaling action in HSC<sup>[59]</sup>, are all potential targets for treatment. There are no clinical trials to date involving these miRNAs as therapeutic targets in ALD and further studies will be necessary before clinical application.

## **ROLE OF MIRNAS IN NAFLD**

NAFLD is defined as the accumulation of fat in the liver in the absence of alcohol intake, viral infection or other specific causes of liver disease. NAFLD represents a spectrum of disorders ranging from the simple accumulation of triglycerides in hepatocytes (hepatic steatosis) to steatosis with inflammation [non-alcoholic steatohepatitis (NASH)], fibrosis and cirrhosis<sup>[60]</sup>. NAFLD and NASH have rapidly become the most common cause of chronic liver disease worldwide in recent decades. The prevalence of these diseases has been estimated between 25% to 45% of the general population<sup>[61]</sup> with a greater prevalence in patients with obesity, diabetes mellitus or metabolic syndrome, in which case, the

prevalence of NAFLD can reach 70% to 90%<sup>[62-64]</sup>. It is estimated that by 2020 cirrhosis related to NAFLD will be the first indication for liver transplantation<sup>[65]</sup>.

### Pathogenic role of miRNAs in NAFLD

The pathogenesis of NAFLD, along with the underlying mechanisms of progression from steatosis to steatohepatitis, has not been fully elucidated. Traditionally, the “two hit” theory<sup>[66]</sup> has been upheld. The “first hit”, which includes insulin resistance leading to the accumulation of fat in the liver, is followed by a “second hit”, consisting of the interaction of inflammatory cytokines, mitochondrial dysfunction and oxidative stress, leading to hepatocellular injury, inflammation and fibrosis<sup>[67]</sup>. However, more recently, multiple factors have been implicated in the pathogenesis of NAFLD, such that the “two hit” theory has been replaced by a “multiple-hit” hypothesis<sup>[68]</sup>. The “multiple-hit” theory includes the involvement of insulin resistance, adipose tissue dysfunction, mitochondrial dysfunction, endoplasmic reticulum stress, dietary factors, fatty acids, iron overload, inflammatory activation, LPS produced by gut microbiota, a chronic inflammatory state, and genetic and epigenetic factors in the pathogenesis and progression of NAFLD<sup>[68-70]</sup>. Accordingly, the following is a summary of the research implicating several miRNAs in the regulation of key targets in the development of NAFLD<sup>[8]</sup>. It is of special interest that recent studies have reported differences in miRNA expression between liver samples from patients with NAFLD and controls. Specifically, livers from patients with NAFLD express an upregulation of miR-31, miR-33a, miR-34a, miR-144, miR-146b, miR-150, miR-182, miR-183, miR-200a, miR-224, and miR-301a and a down regulation of miR-17, miR-122, miR-296, miR-373, miR-375 and miR-378c<sup>[71-76]</sup>. Among these miRNAs, miR-34a, miR-122, and miR-155 have been most often associated with the pathogenesis of NAFLD and as such, the following is a review of these miRNAs in detail. Table 2 displays a list of all miRNAs that have been associated with NAFLD through February 2018.

**miR-122:** miR-122 is the most abundant miRNA in the liver and plays a fundamental role in liver physiology<sup>[77-79]</sup> and lipid metabolism<sup>[80]</sup>. miR-122 interacts with multiple important lipogenic factors in human NAFLD, such as acetyl coA carboxylase-2 (ACC2) and the sterol regulatory element binding protein (SREBP)<sup>[71,81,82]</sup>. miR-122 is decreased in liver samples<sup>[83-85]</sup> but increased in serum<sup>[84,86,87]</sup> from patients with NAFLD compared to healthy controls. Despite this somewhat paradoxical finding, the association of miR-122 with NAFLD pathogenesis is well established. Inhibition of miR-122 in high-fat fed mice is associated with a significant reduction in hepatic steatosis and plasma cholesterol levels, which was associated with a reduction in hepatic sterol and fatty acid synthesis rates and stimulation of hepatic fatty-acid oxidation mediated by activation of

adenosine 5'-monophosphate-activated protein kinase (AMPK)<sup>[80]</sup>. Moreover, the relationship of miR-122 with the development and progression of hepatic fibrosis has been demonstrated *in vitro*, through the regulation of HSC proliferation and production of collagen by targeting prolyl 4-hydroxylase subunit  $\alpha$ -1 (P4HA1)<sup>[88]</sup>.

**miR-34a:** miR-34a is overexpressed in both murine models of NAFLD (e.g., mice fed a high-fat diet) and liver and serum from patients with NAFLD<sup>[81,87,89,90]</sup>. The main target of miR-34a is Sirtuin 1 (SIRT1), which regulates energy homeostasis by activating transcription factors such as peroxisome proliferator activated receptors (PPAR)  $\alpha$  and liver X receptor (LXR). In addition, SIRT1 inhibits the co-activator 1 $\alpha$  of the PPAR- $\gamma$  (PGC1- $\alpha$ ), the SREBP-1c and the farnesoid X receptor (FXR). SIRT1 is downregulated in the liver of NAFLD patients<sup>[91]</sup> and the inhibition of miR-34a restores the expression of SIRT1 and PPAR- $\alpha$ , leading to the activation of AMP-activated protein kinase (AMPK) and several target genes of PPAR- $\alpha$ . These findings suggest a fundamental role for miR-34a in the dysregulation of lipid metabolism associated with NAFLD<sup>[92]</sup>.

**miR-155:** miR-155 is an important regulator of immune cells in both humans and mice and is involved in several inflammatory processes, such as rheumatic diseases<sup>[93]</sup>, lipid metabolism<sup>[94]</sup> and in ALD (as described above). In patients with NAFLD, miR-155 is dysregulated by adipogenic transcription factors CCAAT/enhancer binding protein (C/EBP)- $\alpha$ , C/EBP- $\beta$ , PPAR- $\gamma$  and LXR $\alpha$ <sup>[95,96]</sup>, fibrosis targets platelet derived growth factor (PDGF), Smad3 and C/EBP- $\beta$ <sup>[97]</sup>, and a tumor suppressor in the liver, SOCS-1<sup>[90,98]</sup>. However, animal models of NAFLD show contradictory results. For example, miR-155 deficient mice fed a high-fat diet showed a significant increase in hepatic steatosis<sup>[98]</sup>, while miR-155 KO mice fed a methionine-choline-deficient diet showed a decrease in steatosis and expression of genes involved in fatty acid metabolism and fibrosis, with no concomitant liver injury or inflammation<sup>[97]</sup>. In addition, miR-155 may also be involved in hepatocarcinoma development<sup>[99]</sup>. These findings suggest that miR-155 may have different roles in fat storage and lipid accumulation in liver diseases and healthy subjects. However, additional research is warranted<sup>[97]</sup>.

### miRNAs as biomarkers in the diagnosis of NAFLD

As shown in Table 2, many miRNAs are differentially expressed in patients with NAFLD compared to healthy controls. These miRNAs may serve as potential biomarkers in the diagnosis and staging of NAFLD.

**miR-122:** Several studies have found that miR-122 is elevated in serum in NAFLD patients<sup>[81,86,100-102]</sup>, even long before an alteration in transaminase levels occurs<sup>[103]</sup>. The diagnostic potential of miR-122 may extend to an indicator of disease severity and as a



**Table 2** Summary of microRNAs associated with non-alcoholic fatty liver disease

| miRNA  | Source of samples          | Change        | Main targets                         |
|--|----------------------------|---------------|--------------------------------------|
| miR-9 <sup>[129]</sup>                                       | Human serum;               | Upregulated   | Oncut2; SIRT1                        |
| miR-10b <sup>[130]</sup>                                     | Human hepatocyte cell line | Downregulated | PPAR $\alpha$                        |
| miR-15b <sup>[131,132]</sup>                                 | Animal models              | Upregulated   |                                      |
| miR-16 <sup>[104]</sup>                                      | Human serum                | Upregulated   |                                      |
| miR-17 <sup>[74]</sup>                                       | Human liver                | Downregulated |                                      |
| miR-19 <sup>[84]</sup>                                       | Human serum                | Upregulated   |                                      |
| miR-21 <sup>[86,87,99,133-136]</sup>                         | Animal models              | Upregulated   | PPAR $\alpha$ ; TGF- $\beta$         |
|  | Human hepatocyte cell line |               | PTEN                                 |
|  | Human liver and serum      |               |                                      |
| miR-21 <sup>[85,89,137,138]</sup>                            | Animal models              | Downregulated | HMGCR; FABP7                         |
|  | Human liver                |               |                                      |
| miR-24 <sup>[139]</sup>                                      | Human hepatocyte cell line |               |                                      |
|  | Animal models              | Upregulated   | Insig1; SREBP                        |
|  | Human hepatocyte cell line |               |                                      |
| miR-26 <sup>[140]</sup>                                      | Animal models              | Downregulated | IL-6, IL-7                           |
| miR-27a <sup>[141]</sup>                                     | Animal models              | Downregulated |                                      |
| miR-27b <sup>[102]</sup>                                     | Human serum                | Upregulated   |                                      |
| miR-29a <sup>[142,143]</sup>                                 | Animal models              | Downregulated | HMGCR; LPL                           |
| miR-29c <sup>[85,89,90]</sup>                                | Animal models              | Downregulated | DNMT3A; DNMT3B                       |
| miR-30b <sup>[83]</sup>                                      | Human liver                | Downregulated | ITGAX; FABP4                         |
|  | Human hepatocyte cell line |               |                                      |
| miR-30c <sup>[144]</sup>                                     | Human serum                | Upregulated   |                                      |
| miR-31 <sup>[74,89]</sup>                                    | Human liver                | Upregulated   |                                      |
|  | Animal models              |               |                                      |
| miR-33a <sup>[73,76]</sup>                                   | Human liver                | Upregulated   | ABCA1; ABCA2                         |
| miR-33a <sup>[85]</sup>                                      | Human liver                | Downregulated |                                      |
| miR-34a <sup>[71,81,82,85,87,89,90,92,104,105,145-148]</sup> | Animal models              | Upregulated   | SIRT1; HNF4 $\alpha$ ; PPAR $\alpha$ |
|  | Human hepatocyte cell line |               |                                      |
|  | Human liver and serum      |               |                                      |
| miR-99a <sup>[149]</sup>                                     | Human serum                | Downregulated |                                      |
| miR-101 <sup>[150]</sup>                                     | Human hepatocyte cell line | Upregulated   | ABCA1                                |
|  | Human monocyte cell line   |               |                                      |
| miR-103 <sup>[53,89,151]</sup>                               | Animal models              | Upregulated   | Cav1                                 |
|  | Human liver and serum      |               |                                      |
| miR-103a <sup>[152]</sup>                                    | Human liver                | Upregulated   |                                      |
|  | Human hepatocyte cell line |               |                                      |
| miR-106b <sup>[152]</sup>                                    | Human liver                | Upregulated   |                                      |
| miR-107 <sup>[53,89]</sup>                                   | Animal models              | Upregulated   | Cav1                                 |
|  | Human liver                |               |                                      |
| miR-122 <sup>[81,84,86,87,101-104,106,153]</sup>             | Animal models              | Upregulated   |                                      |
|  | Human Serum                |               |                                      |
| miR-122 <sup>[71,82-85,89,90,99,106,141,154,155]</sup>       | Animal models              | Downregulated | ACC-2; HAMP; FAS;                    |
|  |                            |               | HMGCR; SREBF-1c                      |
|  | Human liver                |               | SREPB-2; HIF-1 $\alpha$ ;            |
|  |                            |               | Vimentin; MAP3K3                     |
| miR-125b <sup>[84]</sup>                                     | Human serum                | Upregulated   |                                      |
| miR-125b <sup>[156]</sup>                                    | Animal models              | Downregulated | FAS                                  |
| miR-139-5p <sup>[83]</sup>                                   | Human liver                | Downregulated | TNF $\alpha$                         |
| miR-144 <sup>[76]</sup>                                      | Human Liver                | Upregulated   | ABCA1                                |
| miR-144 <sup>[157]</sup>                                     | Animal models              | Downregulated | TLR-2                                |
| miR-146a <sup>[158]</sup>                                    | Animal models              | Upregulated   |                                      |
|  | Human hepatocyte cell line |               |                                      |
| miR-146a <sup>[132]</sup>                                    | Animal models              | Downregulated | Wnt1; Wnt5                           |
|  | Human hepatocyte cell line |               |                                      |
| Mir-146b <sup>[149,159]</sup>                                | Animal models              | Downregulated | IRAK1                                |
|  | Human serum                |               | TRAF6                                |
|  | Human hepatocyte cell line |               |                                      |
| miR-146b <sup>[71,83,158]</sup>                              | Animal models              | Upregulated   |                                      |
|  | Human liver                |               |                                      |
|  | Human hepatocyte cell line |               |                                      |
| miR-149 <sup>[160]</sup>                                     | Animal models              | Upregulated   | FGF-21                               |
|  | Human hepatocyte cell line |               |                                      |
| miR-150 <sup>[74]</sup>                                      | Human liver                | Upregulated   |                                      |
| miR-152 <sup>[158]</sup>                                     | Animal models              | Upregulated   |                                      |
|  | Human hepatocyte cell line |               |                                      |

|   |                            |               |                        |
|---|----------------------------|---------------|------------------------|
| miR-155 <sup>[90,97-99,161,162]</sup>                 | Animal models              | Upregulated   | SOCS1; C/EBP-β; CES3;  |
| miR-155 <sup>[96]</sup>                               | Human hepatocyte cell line |               | PDGF; SMAD3            |
|   | Animal models              | Downregulated | LXRα                   |
|   | Human liver and serum      |               |                        |
| miR-181a <sup>[82]</sup>                              | Animal models              | Upregulated   |                        |
| miR-181d <sup>[149]</sup>                             | Human serum                | Downregulated |                        |
| miR-182 <sup>[74]</sup>                               | Human liver                | Upregulated   | FOXO3                  |
| miR-183 <sup>[74]</sup>                               | Human liver                | Upregulated   |                        |
| miR-192 <sup>[84,90]</sup>                            | Animal models              | Downregulated |                        |
|   | Human liver                |               |                        |
| miR-192-5p <sup>[82,84,86,102,106]</sup>              | Animal models              | Upregulated   |                        |
|   | Human liver and serum      |               |                        |
| miR-194 <sup>[89]</sup>                               | Animal models              | Upregulated   |                        |
| miR-197 <sup>[149]</sup>                              | Human serum                | Downregulated |                        |
| miR-199 <sup>[163]</sup>                              | Animal models              | Upregulated   | Cav1; PPARα            |
|   | Human hepatocyte cell line |               |                        |
|   | Human liver                |               |                        |
| miR-200a/b/c <sup>[74,82,89,90,141,158,162,164]</sup> | Animal models              | Upregulated   | ZEB1; CDH1; EZH2; IRP1 |
|   | Human hepatocyte cell line |               |                        |
| miR-203 <sup>[90,132]</sup>                           | Animal models              | Downregulated |                        |
| miR-212 <sup>[165]</sup>                              | Animal models              | Upregulated   | FGF-21                 |
|   | Human hepatocyte cell line |               |                        |
| miR-214 <sup>[71,166]</sup>                           | Human liver                | Upregulated   |                        |
|   | Animal models              |               |                        |
| miR-216 <sup>[167]</sup>                              | Animal models              | Downregulated |                        |
| miR-219a <sup>[74]</sup>                              | Human liver                | Downregulated |                        |
| miR-221 <sup>[73]</sup>                               | Human liver                | Downregulated |                        |
| miR-221 <sup>[89,90,99]</sup>                         | Animal models              | Upregulated   |                        |
| miR-222 <sup>[99]</sup>                               | Animal models              | Upregulated   |                        |
| miR-223 <sup>[86,164]</sup>                           | Animals models             | Upregulated   | IRP1                   |
|   | Human serum                |               |                        |
| miR-224 <sup>[73,74]</sup>                            | Human liver                | Upregulated   |                        |
| miR-291b <sup>[168]</sup>                             | Animal models              | Upregulated   | AMPKα1                 |
| miR-302a <sup>[167]</sup>                             | Animals model              | Downregulated | ELOVL6                 |
| miR-331 <sup>[144]</sup>                              | Human serum                | Upregulated   |                        |
| miR-335 <sup>[89]</sup>                               | Animal models              | Upregulated   |                        |
| miR-375 <sup>[84]</sup>                               | Human serum                | Upregulated   |                        |
| miR-378 <sup>[74]</sup>                               | Human liver                | Downregulated |                        |
| miR-421 <sup>[169]</sup>                              | Animal models              | Upregulated   | SIRT-3                 |
| miR-422a <sup>[83]</sup>                              | Human liver                | Downregulated |                        |
| miR-429 <sup>[141]</sup>                              | Animal models              | Upregulated   |                        |
| miR-451 <sup>[87]</sup>                               | Human Serum                | Upregulated   |                        |
| miR-451 <sup>[89,141,170]</sup>                       | Animal models              | Downregulated | AMPK/ AKT              |
|   | Human liver                |               |                        |
| miR-467b <sup>[171]</sup>                             | Animal models              | Downregulated | LPL                    |
| miR-576 <sup>[152]</sup>                              | Human liver                | Downregulated | RAC1                   |
|   | Human hepatocyte cell line |               |                        |
| miR-590 <sup>[74]</sup>                               | Human liver                | Downregulated |                        |
| miR-892a <sup>[152]</sup>                             | Human liver                | Upregulated   |                        |
|   | Human hepatocyte cell line |               |                        |
| miR-1290 <sup>[102]</sup>                             | Human serum                | Upregulated   |                        |

Onecut2: One cut homeobox 2; SIRT: Sirtuin; PPARα: Peroxisome proliferator activated receptor α; TGF-β: Transforming growth factor β; PTEN: Phosphatase and tensin homolog; HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase; FABP: Fatty acid binding protein; Insig1: Insulin induced gene 1; SREBP: Sterol regulatory element binding protein; IL: Interleukin; LPL: Lipoprotein lipase; DNMT: DNA methyltransferase; ITGAX: Integrin subunit α X; ABCA: ATP binding cassette subfamily A; HNF4α: Hepatocyte nuclear factor 4 α; Cav1: Caveolin 1; ACC-2: Acetyl-CoA carboxylase 2; SREBF: Sterol regulatory element binding transcription factor; HIF-1α: Hypoxia inducible factor 1 α; MAP3K3: Mitogen-activated protein kinase kinase kinase 3; FAS: Fatty acid synthase; TNFα: Tumour necrosis factor α; TLR-2: Toll-like receptor 2; Wnt: Wnt family member; IRAK1: Interleukin 1 receptor associated kinase 1; TRAF6: TNF receptor associated factor 6; FGF-21: Fibroblast growth factor 21; SOCS1: Suppressor of cytokine signaling 1; C/EBPβ: CCAAT/enhancer binding protein β; CES3: Carboxylesterase 3; PDGF: Platelet derived growth factor; SMAD3: SMAD family member 3; LXRα: Liver X receptor; FOXO3: Forkhead box O3; ZEB-1: Zinc finger E-box binding homeobox 1; CDH1: Cadherin 1; EZH2: Enhancer of zeste 2 polycomb repressive complex 2; IRP1: Iron regulatory protein 1; AMPKα1: AMPK: Adenosine monophosphate activated protein kinase α 1; ELOVL6: ELOVL fatty acid elongase 6; AMPK: Adenosine monophosphate activated protein kinase; AKT: AKT serine/threonine kinase 1; RAC1: Ras-related C3 botulinum toxin substrate 1.

predictor of hepatic fibrosis<sup>[82,84,87,104]</sup>.

**miR-34a:** Similar to miR-122, miR-34a has also been shown to have potential as a biomarker of diagnosis and

severity of NAFLD. Several studies have shown that miR-34a is upregulated in the liver and serum of patients with NAFLD<sup>[71,81,82,104]</sup>. Additionally, elevated serum levels of miR-34a correlate with disease severity from simple

steatosis to steatohepatitis, with liver enzyme levels, with fibrosis stage and with inflammation activity<sup>[82,104,105]</sup>.

**miR-192:** Serum miR-192 levels are positively correlated with the severity of NAFLD-specific liver pathomorphological changes in mice fed a choline and folate deficient diet<sup>[82]</sup> and miR-192 upregulation in human serum has been demonstrated<sup>[82,84,86,102,106]</sup>. Interestingly, serum levels of miR-122 and miR-192 have been shown to be strongly correlated<sup>[84,86]</sup>.

**Panels:** In addition to individual miRNAs, a serum panel comprised of hsa-miR-122-5p, hsa-miR-1290, hsa-miR-27b-3p, and hsa-miR-192-5p has shown high NAFLD diagnostic accuracy, regardless of NAFLD activity score (NAS) status<sup>[102]</sup>. Another research group found that NAFLD was associated with an miRNA signature based on up-regulation of miR-122, miR-192, miR-19a, miR-19b, miR-125b, and miR-375<sup>[84]</sup>.

It is important to mention that most studies have compared patients with NAFLD to healthy controls or patients with chronic viral hepatitis B<sup>[105]</sup> or C<sup>[104]</sup>. However, no comparisons have been performed, to our knowledge, between patients with NAFLD and patients with ALD.

#### Therapeutic application of miRNAs in NAFLD

As previously mentioned, miRNAs are involved in several stages of NAFLD development (from lipid metabolism or diabetes to liver inflammation), and are therefore potential therapeutic targets<sup>[7,107]</sup>. The expression of miR-103 and miR-107 is upregulated in obese mice<sup>[53,89]</sup>. Inactivation of miR-103/107 in murine adipocytes upregulates caveolin-1 (a critical mediator of the insulin receptor) leading to enhanced insulin signaling, decreased adipocyte size and enhanced insulin-stimulated glucose uptake<sup>[53,108]</sup>. An N-acetylgalactosamine (GalNAc)-conjugated anti-miR-103/107 (RG-125/AZD4076, Regulus Therapeutics) has been developed for the treatment of NAFLD and type 2 diabetes or pre-diabetes<sup>[108-110]</sup>. Currently, two clinical trials are registered using this drug in patients with NAFLD (ClinicalTrials.gov Identifier: NCT02826525 and NCT02612662), although Regulus has acknowledged that AstraZeneca intends to terminate the clinical development of RG-125/AZD4076<sup>[108,111]</sup>.

miR-122 has also shown promising results as a treatment for NAFLD. There is a high concentration of miR-122 in liver tissue<sup>[112]</sup> and this miRNA plays an important role in liver development, differentiation, homeostasis and functioning<sup>[113]</sup>. Over-expression of miR-122 may affect the Ying Yan 1 and Farnesoid X Receptor (YY1-FXR-SHP) regulatory axis leading to a reduction in hepatic triglyceride levels, potentially serving as a target for NAFLD treatment<sup>[114]</sup>. miR-122 is also an essential host factor for hepatitis C virus (HCV) replication and anti-miR-122 efficiently reduces viral load

in chronically infected HCV patients without detectable resistance<sup>[108]</sup>. The fact that miR-122 has protective effects on NAFLD, while imposing a deleterious impact on HCV infection, emphasizes the importance of cautious targeting of miRNAs therapy since the role of miRNAs can be highly context dependent<sup>[115]</sup>.

circRNA\_0046366 antagonizes miR-34a and normalizes PPAR $\alpha$  signaling, leading to the amelioration of liver steatosis in a murine model<sup>[116]</sup>. However, a phase I study on the effects of a miR-34 mimic (MRX34) on primary liver cancer and advanced or metastatic cancer with liver involvement (ClinicalTrials.gov Identifier: NCT01829971) was prematurely terminated due to serious immune-related adverse events<sup>[108]</sup>, highlighting the potential risks of miRNA based-therapies.

## CONCLUSION

All except four (miR-199, miR-212, miR-214 and miR-497) of the 21 miRNAs associated with ALD, listed in Table 1, are also related to NAFLD or lipid metabolism (although the four have been associated with other diseases, such as cancer<sup>[117]</sup>). Conversely, miRNAs that are related to the pathogenesis of NAFLD (miR-122, miR-34a and miR-155) are also clearly linked to ALD. These results reflect the common mechanisms between NAFLD and ALD and also the pleiotropic effects of any particular miRNA.

Due to the lack of specificity of miRNAs, the development of a biomarker or treatment specific to ALD or NAFLD is difficult. It is more feasible that individual miRNAs or a panel of miRNAs would be useful in the staging of liver disease (e.g., distinguishing simple steatosis in ALD or NAFLD from steatohepatitis)<sup>[118]</sup>. miR-122 is the most promising candidate as a biomarker due to its liver specificity. It is clear however, that miR-122 is also a marker of liver damage regardless of etiology<sup>[119]</sup>. Technical limitations, such as standardization of techniques and potential costs, add to the difficulties inherent to the development of a validated diagnostic biomarker. Circulating miRNAs are promising as biomarkers due to their stability and potential ability to detect advanced liver disease without a biopsy. However, rigorously validated studies are needed before they can be brought to the clinic<sup>[119]</sup>.

The development of miRNA-targeted interventions for ALD and NAFLD is an intriguing area of research. However, despite the success in animal models and the potential targets described in this review, to the best of our knowledge there are no current clinical trials for miRNA interventions in ALD or NAFLD. The few studies that are being conducted on miRNA treatment in other diseases are phase 1 studies in the field of cancer research (e.g., assessing the activity of miRNA-loaded minicells or TargomiRs in malignant pleural mesothelioma<sup>[120]</sup>). Theoretical miRNA-based therapies are pharmacologically complex and include miRNA inhibition (e.g., synthetic

anti-miRNAs) or miRNA replacement therapy (e.g., lipid vesicles or gold nanoparticles)<sup>[121]</sup>. One major challenge to the development of miRNA-based therapies is the improvement of drug delivery systems. Due to the biochemical instability of unmodified miRNAs and potential immunogenicity, specific delivery to target organs should be achieved. The high degree of redundancy among miRNAs and the multiple binding sites for any given miRNA must also be taken into account when designing efficacious and safe miRNA-based therapies<sup>[122]</sup>.

To sum up, there is a large body of literature regarding miRNAs in NAFLD and ALD at various stages of the disease. These studies include expression data from microarrays and next generation sequencing from animal models and human studies, and cell-specific data from in situ hybridization and sensor constructs. The role of miRNAs in pathogenesis is well-documented and as such, their potential value as biomarkers or therapeutic targets is warranted. However, most miRNA modifications have a modest phenotypic effect, since miRNAs are unlikely to be the single key factor in chronic and multifactorial diseases such as liver steatosis<sup>[123]</sup>. Instead, most miRNAs act as fine-tuners in disease pathways and this characteristic, along with their lack of specificity must be considered before use in the clinic. To this end, we must improve our understanding of the interaction of different miRNAs in the development of advanced liver disease.

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## Calcium-sensing receptor in colorectal inflammation and cancer: Current insights and future perspectives

Luca Iamartino, Taha Elajnaf, Enikő Kallay, Martin Schepelmann

Luca Iamartino, Taha Elajnaf, Enikő Kallay, Martin Schepelmann, Institute of Pathophysiology and Allergy Research, Medical University of Vienna, Vienna 1090, Austria

ORCID number: Luca Iamartino (0000-0003-0794-3565); Taha Elajnaf (0000-0002-7293-254X); Enikő Kallay (0000-0002-4996-0104); Martin Schepelmann (0000-0002-7017-5426).

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**Correspondence to:** Martin Schepelmann, PhD, Postdoctoral Fellow, Department of Pathophysiology and Allergy Research, Medical University of Vienna, Währinger Gürtel 18-20, Vienna 1090, Austria. [martin.schepelmann@meduniwien.ac.at](mailto:martin.schepelmann@meduniwien.ac.at)  
Telephone: +61-743-14040051230  
Fax: +61-743-14040051300

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### Abstract

The extracellular calcium-sensing receptor (CaSR) is best known for its action in the parathyroid gland and kidneys where it controls body calcium homeostasis. However, the CaSR has different roles in the gastrointestinal tract, where it is ubiquitously expressed. In the colon, the CaSR is involved in controlling multiple mechanisms, including fluid transport, inflammation, cell proliferation and differentiation. Although the expression pattern and functions of the CaSR in the colonic microenvironment are far from being completely understood, evidence has been accumulating that the CaSR might play a protective role against both colonic inflammation and colorectal cancer. For example, CaSR agonists such as dipeptides have been suggested to reduce colonic inflammation, while dietary calcium was shown to reduce the risk of colorectal cancer. CaSR expression is lost in colonic malignancies, indicating that the CaSR is a biomarker for colonic cancer progression. This dual anti-inflammatory and anti-tumourigenic role of the CaSR makes it especially interesting in colitis-associated colorectal cancer. In this review, we describe the clinical and experimental evidence for the role of the CaSR in colonic inflammation and colorectal cancer, the intracellular signalling pathways which are putatively involved in these actions, and the possibilities to exploit these actions of the CaSR for future therapies of colonic inflammation and cancer.

**Key words:** Calcium-sensing receptor; Colon; Cancer; Inflammation; Calcimimetics; Calcilytics

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**Core tip:** The extracellular calcium-sensing receptor (CaSR) is best known for its roles in maintaining body calcium homeostasis, but it is also expressed in the intestines where it is assumed to be involved in pathologies such as inflammatory bowel disease and colorectal cancer. It has been suggested to act as a tumour suppressor in colorectal tumourigenesis. In this review we highlight the evidence for the anti-inflammatory and anti-tumourigenic roles of the CaSR, its signalling pathways, and its potential for future use as a drug target in the context of inflammatory bowel disease and colorectal cancer.

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## INTRODUCTION

### Extracellular calcium-sensing receptor

The extracellular calcium-sensing receptor (CaSR) was first identified in bovine parathyroid cells. It is a G protein-coupled receptor (GPCR) that is activated by extracellular calcium ( $\text{Ca}^{2+}$ ), which acts as a first messenger of the CaSR signalling cascade<sup>[1]</sup>. The main physiological role of the CaSR is to control serum  $\text{Ca}^{2+}$  levels through regulating the synthesis and secretion of parathyroid hormone (PTH), which acts directly on the kidneys, bones and indirectly on the intestines to maintain normocalcaemia<sup>[2]</sup>. Therefore, the CaSR acts as a "calciostat" which maintains serum  $\text{Ca}^{2+}$  concentration within a tight range (1.1-1.3 mmol/L free ionised  $\text{Ca}^{2+}$ ) and is expressed in calcitropic tissues, such as parathyroid glands, kidneys and bone. In addition to its pivotal role in maintaining serum  $\text{Ca}^{2+}$  homeostasis, the CaSR also regulates non-calcitropic functions, such as gene expression, smooth muscle contraction, differentiation, proliferation, inflammation, and ion channel activity in other tissues, such as the colon, liver, vasculature, lung, pancreas, brain and the placenta<sup>[3-6]</sup>. The CaSR is also expressed along the entire gastrointestinal (GI) tract and regulates various functions in the intestines. These include dual regulation of fluid transport, where it stimulates  $\text{Cl}^-$  and short chain fatty acid-dependent  $\text{HCO}_3^-$  secretion, but inhibits cyclic adenosine monophosphate (cAMP)-dependent  $\text{HCO}_3^-$  secretion<sup>[7]</sup>. In addition, the CaSR is expressed in the myenteric plexi where it regulates gut motility<sup>[8]</sup>. It also acts as a nutrient sensor for digestion products<sup>[9]</sup>, such as amino acids. Additionally, it plays a role in intestinal inflammation and in the maintenance of gut microbiota and immune homeostasis<sup>[10]</sup>.

The CaSR is a multifaceted GPCR that couples to several heterotrimeric G proteins. It modulates signalling pathways downstream of  $\text{G}_{q/11}$ ,  $\text{G}_{i/o}$ ,  $\text{G}_{12/13}$ <sup>[11]</sup> and in specific cell contexts  $\text{G}_s$ <sup>[12]</sup>. Signalling output by the CaSR is also ligand-dependent as well as cell-type specific, thus adding to the diversity of the CaSR-mediated signalling pathways. Table 1<sup>[5,16-26]</sup> shows examples of both naturally occurring and synthetic CaSR ligands and their reported direct effects on inflammation and cancer *in vivo*.

Mutations in the *CASR* gene result in  $\text{Ca}^{2+}$  homeostasis-related diseases, including familial hypocalciuric hypercalcaemia (FHH1) and neonatal severe hyperparathyroidism (NSHPT), both of which are caused by inactivating mutations, as well as autosomal dominant hypocalcaemia (ADH1), which is caused by activating mutations (for review see<sup>[27]</sup>). Such disease causing mutations result in altered signalling output by the receptor and/or reduced cell surface expression<sup>[28]</sup>. In the intestines, more focus is directed towards the CaSR as a therapeutic target for intestinal diseases including diarrhoea, inflammatory bowel disease and colorectal cancer. In the colon, loss of CaSR expression is associated with colonic tumourigenesis<sup>[29]</sup>. In addition, clinical trials show that  $\text{Ca}^{2+}$  intake can favourably modulate normal colon tissue and circulating inflammation biomarkers for risk of colorectal neoplasms in sporadic colorectal adenoma patients<sup>[17]</sup>. This has led to the hypothesis that the CaSR plays a role in cancer prevention. In the following sections, we highlight the role of the CaSR in intestinal inflammation and colorectal cancer.

## ROLE OF THE CaSR IN INFLAMMATION

The CaSR is expressed in a wide range of inflammation-associated cell types where it regulates various functions. It is expressed in immune cells including macrophages, eosinophils and monocytes<sup>[5,30,31]</sup>. In these CaSR-expressing human and murine circulating monocytes, extracellular  $\text{Ca}^{2+}$  induces a chemokinetic effect<sup>[32]</sup>. The CaSR is also implicated in immune regulation where it plays a dual role: as a responder to inflammatory cytokine release on the one hand, and as a promoter of inflammation on the other. The link between the CaSR and inflammation has been explored in several studies. *In vitro*, inflammatory cytokines upregulate the CaSR expression in various cell types through defined response elements on the *CASR* gene<sup>[33,34]</sup>. *In vivo* studies also suggest a link between inflammatory cytokines and the CaSR, as intraperitoneal injection of IL-1 $\beta$  and IL-6 reduced PTH and 1,25(OH) $_2\text{D}_3$  levels followed by a decrease in serum  $\text{Ca}^{2+}$ <sup>[33,35]</sup>. Furthermore, clinical studies show that hypocalcaemia occurs in critically ill patients where plasma inflammatory cytokines levels are increased<sup>[36]</sup>. In addition, the expression of the CaSR is increased in monocytes from rheumatoid arthritis patients with severe coronary artery calcification<sup>[37]</sup>.

**Table 1** Examples of orthosteric agonists and allosteric modulators of the calcium-sensing receptor<sup>[13-15]</sup>

| Ligand type   | Class and examples  | Reported effects on inflammation  | Reported effects on cancer   | Ref.      |
|---|---|---|--|-----------|
| Orthosteric agonists                                  | Inorganic divalent and trivalent cations: Zn <sup>2+</sup> , Ca <sup>2+</sup> ; Mg <sup>2+</sup> ; Gd <sup>3+</sup> | Reduces inflammation in mouse models of colitis<br>Intake is correlated with reduced inflammation | High Ca <sup>2+</sup> intake: Associated low risk for CRC  | [16-18]   |
|   | Polyamines: Spermine spermidine, putrescine   | Increase airway inflammation and hyperresponsiveness  | Reduce pancreatic cancer growth in mice  | [5,19]    |
|   | Aminoglycoside antibiotics: Neomycin, gentamycin, tobramycin  | -   | -  |           |
|   | Basic polypeptides: poly-L-arginine, L-poly-L-lysine, and amyloid $\beta$ -peptides                                 | Induces airway inflammation<br>Reduces inflammation in mouse models of colitis                    | -  | [5,20]    |
| Combined orthosteric and allosteric modulators        | D-amino-acid polypeptides: Etelcalcetide  | -   | -  |           |
|   | L-amino acids: Phenylalanine, tryptophan  | -   | -  |           |
|   | Glutamyl dipeptides: L-Glu-Val, L-Glu-Cys   | Reduces inflammation in mouse models of colitis   | -  | [21]      |
| Allosteric modulators (calcimimetics and calcilytics) | Small molecule calcimimetics: Sensipar (1Cinacalcet HCl), NPS-R568, GSK3004774                                      | Increases airway hyperresponsiveness  | Treatment of parathyroid tumours<br>Inhibits neuroblastoma tumour growth<br>Reduces hypercalcaemia of malignancy | [5,22-24] |
|   | Small molecule calcilytics: NPS-2143, Calhex, Ronacalaret, AXT-914  | Reduces pulmonary inflammation and airway hyperresponsiveness in rodents                          | -  | [5,25,26] |

<sup>1</sup>Indicates the compounds for which the *in vivo* effects were reported. While many of these modulators have been reported to have *in vitro* effects on (cancer) cell lines, evidence of their *in vivo* activity has remained scarce. The table summarises their known (putatively) CaSR-mediated direct effects on inflammation and cancer in humans or animals. CRC: Colorectal cancer.

### Inflammatory pathways regulated by the CaSR

The CaSR regulates diverse and intricate signalling networks and this regulation is tissue-, and ligand-dependent. In murine macrophages, the CaSR activates the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome through a mechanism that involves increased intracellular Ca<sup>2+</sup> and decreased cAMP levels<sup>[38]</sup>. Moreover, the CaSR regulates polymorphonuclear neutrophil function through a mechanism that likely involves the NF $\kappa$ B pathway<sup>[39]</sup>. The mechanism by which L-tryptophan, L-valine and glutamyl dipeptides mediate the CaSR-dependent inhibition of pro-inflammatory cytokine secretion in colonocytes appears to require  $\beta$ -arrestin 2<sup>[20,21]</sup>. Moreover, in the thick ascending limb of the kidneys, the CaSR has been shown to induce TNF- $\alpha$ -dependent cyclooxygenase 2 expression and prostaglandin E<sub>2</sub> synthesis *via* a Gi-dependent mechanism<sup>[40]</sup>. However, the exact mechanism by which the CaSR regulates inflammation is still unclear and needs further investigation.

### Tissue-specific roles of the CaSR in inflammation

Interestingly, regulation of inflammation by the CaSR appears to be tissue-dependent. One example is the pivotal role of the CaSR in airway hyperresponsiveness and inflammation in allergic asthma. Studies on mice show that the calcilytic NPS-2143 ameliorates the severity of allergen-induced airway hyperresponsiveness<sup>[5]</sup>. In agreement with that, NPS-2143 was also shown

by an independent group to be protective against lipopolysaccharide-induced pulmonary inflammation<sup>[26]</sup> and against inflammation caused in cigarette smoke extract-stimulated airway epithelial cells<sup>[25]</sup>. The CaSR plays a pro-inflammatory role also in human adipose cells and adipose tissue, where it induced the expression of inflammatory cytokines<sup>[41]</sup>. Paradoxically, in the intestines the CaSR has been suggested by several studies to play an anti-inflammatory role. Below, we highlight the evidence for the anti-inflammatory effects of the CaSR in the intestines and the potential to exploit it for nutraceutical and pharmaceutical intervention.

## CaSR IN INTESTINAL INFLAMMATION

### *In vivo* anti-inflammatory effects of the intestinal CaSR

Evidence supporting the role of the CaSR in intestinal inflammation comes from a study in an intestinal epithelial cell-specific CaSR knock-out mouse model. This study showed that deletion of the CaSR from the intestinal epithelial cells diminished intestinal barrier integrity, altered the composition of the gut microbiota and induced stimulatory inflammatory responses<sup>[42]</sup>. These intestine specific CaSR knock-out mice were more susceptible to dextran sulphate sodium (DSS)-induced inflammation leading to colitis, which is a model for chemically induced inflammation in rodents. *Ex vivo* assessment of intestinal permeability revealed that in the knock-out mice the paracellular transport pathway



was impaired. Consistent with that observation, the colonic expression of tight junction proteins, particularly claudin-2, was reduced in knock-out mice, while the expression of myosin light-chain kinase-1, an enzyme that controls contractility of the perijunctional actomyosin rings and epithelial permeability, was significantly increased<sup>[42]</sup>. No significant differences were seen between the overall richness and diversity of the gut microbiota of knock-out and wild type littermates, yet the bacterial composition was significantly changed. Moreover, intestine specific CaSR knock-out mice had significantly lower epithelial expression of Reg3 $\beta$  and Reg3 $\gamma$  that encode secreted C-type lectins which bind and protect against translocation and dissemination of bacteria. Furthermore, gene array analysis revealed increased expression of inflammatory cytokines including IL-1R in the distal colons of the intestinal epithelium-specific CaSR knock-out mice, as well as in their colonic CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes. In addition, a marked increase in NF $\kappa$ B-dependent genes was observed in the knock-out mice. The expression of programmed cell death protein 1 (PD-1) was significantly enhanced in colonic CD4<sup>+</sup> and CD8<sup>+</sup> T cells<sup>[42]</sup>.

Similarly, recent studies support the anti-inflammatory role of the CaSR in a DSS-colitis mouse model, where poly-L-lysine and glutamyl dipeptides, orthosteric agonists of the CaSR, reduced inflammation. These anti-inflammatory effects were suggested to be dependent on the CaSR, as their effect was reduced by the intravenous administration of the calcilytic NPS-2143<sup>[20,21]</sup>. Whether this inhibition of the anti-inflammatory effects was due to the systemic actions of the calcilytic or due to a direct action of the drug at the inflamed tissue is yet unknown. Studies assessing whether the expression level of the CaSR is affected by the chronic inflammation of the intestine in human patients suffering from inflammatory bowel disease are still outstanding.

### **Mechanisms by which the CaSR putatively modulates colonic inflammation**

Studies on colon cancer cell lines using CaSR agonists and allosteric modulators suggested that the CaSR influences the production of inflammatory cytokines induced by tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ). L-tryptophan and L-valine inhibited interleukin 8 (IL-8) secretion in both Caco-2 and HT-29 colon cancer cell lines. This effect was reversed by the calcilytic NPS-2143<sup>[20]</sup>. In addition, glutamyl dipeptides inhibited pro-inflammatory cytokines and chemokines including IL-8, IL-6, and IL-1 $\beta$ , while increasing the expression of the anti-inflammatory IL-10 in Caco-2 cells<sup>[21]</sup>. However, it was reported that the CaSR is not detectable in colon cancer cell lines, such as HT-29, which is also supported by evidence from independent studies indicating the scarcity of the CaSR in colon cancer tissue and cell lines<sup>[43]</sup>. Therefore, further validation is needed to confirm whether these anti-inflammatory effects are actually mediated *via* the CaSR. Of note, inflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6, increased the

expression of the CaSR at the mRNA and protein level in some colon cancer cell lines<sup>[34]</sup>. This was suggested to be a defence mechanism against inflammation in the intestines. However, this explanation will have to be carefully validated, as *e.g.*, in lung epithelium, CaSR expression is also increased in the inflamed tissue. There however, the increase (and indeed the CaSR itself) represent a rather pro-inflammatory mechanism, as inhibition of the CaSR markedly reduced airway inflammation and hyperresponsiveness<sup>[5]</sup>.

Given that inflammation is a high risk factor for colorectal cancer, it is imperative to ask the following question: is there a causal relationship between activation of the CaSR, reduced inflammation and the prevention of colorectal cancer? As of yet, this question remains unanswered. It is unclear whether dietary or pharmacological activation of the CaSR in the GI tract prevents inflammation in humans. It is also still unclear whether loss of the CaSR in colorectal tumours correlates with loss of its proposed anti-inflammatory effects. Moreover, it is noteworthy that the presence of inflammatory cytokines in the GI tract and their effect on the expression and/or function of the CaSR add to the complexity of the scenario *in vivo*. Nonetheless, inflammation is a key risk factor for colorectal cancer<sup>[44,45]</sup>, thus targeting the CaSR for mitigating inflammation may very well contribute to colorectal cancer prevention in one fell swoop. Below, we summarise the evidence for the involvement of the CaSR in cancer and specifically colorectal cancer as well as its potential as a therapeutic target.

## **ROLE OF THE CaSR IN CANCER**

The CaSR plays a ying-yang role in tumours: while it is suggested to be an oncogene in breast and prostate tumours, in parathyroid, neuroblastoma and colorectal cancers it acts as a tumour-suppressor<sup>[46-48]</sup>. The CaSR signals *via* multiple signalling pathways and is sensitive to many ligands, the bioavailability of which varies among tissues. The different ligands and different signalling pathways can generate a tissue-specific CaSR response, justifying this dual behaviour during cancer development. Table 2<sup>[3,19,29,49-72]</sup> summarises the different roles of the CaSR in various types of cancer.

### **CaSR acts both as oncogene and tumour suppressor**

The CaSR was implicated in the promotion of metastases from breast, prostate, and kidney tumours, thus acting as an oncogene in these tissues. Its oncogenic role is often mediated by parathyroid hormone related peptide (PTHrP).

Breast cancer has a tendency to form metastases in particular in the bones<sup>[73]</sup>. Metastases originated from breast tumours promote bone resorption which, in turn, causes the release of trophic factors (*e.g.*, TGF- $\beta$  and IGF1) that stimulate tumour cell growth, thus forming a vicious cycle. Osteolysis is driven by osteoclasts that are activated by PTHrP, which is synthesised and

**Table 2** Dual function of the calcium-sensing receptor as tumour suppressor and oncogene in various cancers and the affected calcium-sensing receptor-coupled signalling pathways

| Cancer type     | CaSR              | Expression of the CaSR                                     | Detection     | Proposed signalling pathway   | Ref.         |
|-----------------|-------------------|--|---------------|---|--------------|
| Gastric         | Oncogene          | Increased  | mRNA, protein | TRPV4   | [49]         |
| Prostate        | Oncogene          | Increased  | mRNA, protein | PTHrP <i>via</i> trans-activation of the EGFR and ERK1/2 phosphorylation<br>AKT phosphorylation | [50-52]      |
| Breast          | Oncogene          | Increased in breast primary tumours and in bone metastases | mRNA, protein | PTHrP <i>via</i> cAMP<br>ERK1/2 and TRPC1<br>Inhibition of OPG <i>via</i> epiregulin            | [53-57]      |
| Renal carcinoma | Oncogene          | Increased in bone metastasising tumours                    | mRNA, protein | AKT phosphorylation   | [58]         |
| Colorectal      | Tumour suppressor | Reduced  | mRNA, protein | Canonical and non-canonical Wnt/ $\beta$ -catenin pathway and EMT                               | [3,29,59-62] |
| Endometrial     | Tumour suppressor | Reduced  | Protein       | Apoptosis<br>Wnt/ $\beta$ -catenin<br>VEGFR3  | [63]         |
| Parathyroid     | Tumour suppressor | Reduced  | mRNA, protein | Caveolin-1 and Gαq<br>Cyclin D1 and RGS5  | [64-69]      |
| Neuro-blastoma  | Tumour suppressor | Reduced  | mRNA, protein | Apoptosis <i>via</i> ERK1/2<br>Cancer testis antigens (CTAs)                                    | [22,70,71]   |
| Pancreatic      | Unknown           | Reduced  | mRNA, protein | NCX1/Ca <sup>2+</sup> / $\beta$ -catenin  | [19,72]      |

released from breast cancer cells<sup>[3,74,75]</sup>. The CaSR, highly expressed in metastatic breast cancer cells<sup>[53]</sup>, stimulates PTHrP release, contributing thereby to bone degradation<sup>[54]</sup>. A recent study revealed that cancer cells overexpressing the CaSR had a higher osteolytic potential compared with untransfected cells<sup>[57]</sup>. Therefore, the CaSR could be a predictive marker for bone metastasis and for the patient's poor prognosis.

Like breast cancers, prostate neoplastic lesions have a high capacity to form metastasis in the bone. Highly aggressive prostate cancer cells, such as PC-3, express the CaSR<sup>[50]</sup> while there is no evidence of CaSR expression in normal prostate tissue<sup>[3]</sup>. A cohort study, analysing 1241 prostate cancer patients, found that expression of the CaSR correlated positively with tumour lethality<sup>[76]</sup>.

Although dietary calcium has been suggested to have beneficial effects on the digestive tract as being preventative against colorectal cancer, a recent study pointed out a controversial effect of calcium on gastric cancer development. Xie *et al.*<sup>[49]</sup>, have shown that calcium-activated CaSR promoted gastric cancer cell proliferation and metastasis. Thus, CaSR is suggested to act as an oncogene in the upper part of the gastro-intestinal tract, whereas it seems to act as a tumour suppressor in the lower gastro-intestinal tract (see below) although further studies are required to confirm this hypothesis.

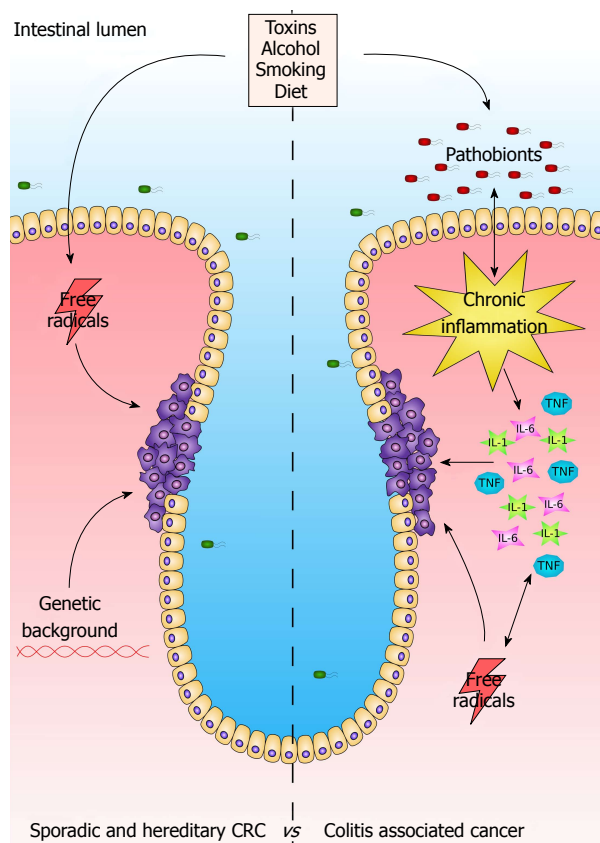
In other cancers like parathyroid cancers, neuroblastoma and colorectal cancer the receptor acts as a tumour suppressor. In parathyroid tumours CaSR expression is inversely correlated with tumour development. CaSR mRNA expression is reduced in parathyroid adenomas and hyperplasias as compared

with normal parathyroid tissue and it is lost in parathyroid carcinoma<sup>[65]</sup>. In the nervous system, the CaSR is expressed during the differentiation of neurons and glial cells<sup>[77,78]</sup>. In neuroblastoma, CaSR expression is positively correlated with neuroblast differentiation and low clinical risk, while undifferentiated and malignant neuroblastomas are CaSR-negative<sup>[79]</sup>. Indeed, ectopic re-expression of the CaSR in MYCN-amplified neuroblastoma cells, which are normally CaSR negative, reduced xenograft growth<sup>[71]</sup>. In addition, treatment with cinacalcet, a positive allosteric modulator of the CaSR, was able to induce the expression of differentiation markers, to inhibit cell proliferation *in vitro* and the growth of mouse tumour xenografts *in vivo*<sup>[22]</sup>.

Another organ in which the CaSR acts as tumour suppressor is the colon.

## CaSR IN COLORECTAL CANCER

The physiological role of the colon is to process and absorb undigested nutrients, absorb electrolytes and water, and to excrete waste products *via* the rectum. As it is a highly renewable tissue, it is prone to malignant transformation. Colorectal cancer (CRC) is one of the most recurrent types of malignancies in the western countries and accounts for over 1,2 million of new cases per year<sup>[80]</sup>. Colorectal tumorigenesis is a complex mechanism developing from the alteration of different molecular processes that control gene expression, cell cycle and apoptosis, which are affected by genetic (*e.g.*, APC mutation), environmental (*e.g.*, diet, alcohol abuse, cigarette smoking, *etc.*), microbial and inflammatory cues that either activate oncogenes or repress tumour suppressors leading then to tumour



**Figure 1 Scheme of colonic carcinogenesis.** Left: Environmental cues such as toxins, alcohol, smoke and diet can produce free radicals (such as reactive oxygen and nitrogen species) that can damage genomic DNA. Accumulating mutations, in particular in genes that encode for mitogenic, cell cycle or apoptosis factors such as APC, BRAF, KRAS, EGF and p53 can then eventually lead to colon carcinogenesis. Genetic background such as inborn APC mutations (hereditary familial adenomatous polyposis) or other hereditary mutations also predispose towards colon tumourigenesis, although hereditary CRC is rare<sup>[81]</sup>. Right: In addition to their direct noxious effect on the tissue, environmental cues can also alter the microbiotic population of the intestine, promoting the proliferation of pathogenic bacteria (pathobionts). Pathobionts and chronic inflammation are closely related and both induce the expression of pro-inflammatory cytokines that accumulate in the mucosa. Persistent inflammation interferes with cell proliferation and apoptosis processes leading to tumourigenesis and in particular in colitis associated cancer. Inflammation itself also induces the production of free radicals that hamper genome stability and can thus cause tumour development<sup>[82]</sup>. TNF: Tumor necrosis factor; IL: Interleukin; CRC: Colorectal cancer.

development (Figure 1<sup>[81,82]</sup>).

As mentioned above, colonic inflammation is a risk factor for developing colorectal cancer. Chronic intestinal inflammatory diseases such as Crohn's disease and ulcerative colitis often lead to colorectal cancer through a process called colitis-associated carcinogenesis (CAC). Similarly to (spontaneous) CRC, CAC leads to genome instability, targeting tumour suppressors and DNA repair mechanisms. However, CRC and CAC differ for prevalence and sequential-timing of the changes in biomarkers during their pathogenesis<sup>[83]</sup>.

CAC is often accompanied by the alteration of the gut microbiota (dysbiosis). Commensal bacteria (eubionts) help to metabolise undigested food, modulating also the immune system of the digestive tract. On the other hand,

pathogenic bacteria can trigger an immune response that, in the worst case, can lead to chronic colitis and other inflammatory bowel diseases<sup>[84]</sup>. As detailed above, the CaSR has been implicated in affecting gut microbiota, and the expression of inflammatory cytokines and thus might play a protective role against the development of CAC by protecting from the deleterious effects of inflammation.

### Epidemiology

In 1985, a small trial demonstrated for the first time that calcium regulates colonocyte proliferation<sup>[85]</sup>. In the same year, Garland *et al.*<sup>[16]</sup> published a retrospective study showing that diets with high calcium content lower the risk of developing colorectal tumours. In the following years, several cohort studies and animal experiments supported the theory that diets rich in calcium and vitamin D prevent the development of colon hyperplasia and cancer - in contrast to western diets with high fat and low calcium and fibre content<sup>[86-88]</sup>. Meta-analyses have since reported that high  $\text{Ca}^{2+}$  intake (more than 1400 mg/d), independent of its source, lowers the risk of CRC, in particular in the distal colon<sup>[89,90]</sup>. Indeed, the evidence for the protective actions of high levels of dietary calcium intake (dairy products) or calcium supplements was rated to be "probably strong" by the World Cancer Research Fund in its most recent update of 2017<sup>[91]</sup>.

Numerous studies have suggested that there is a close interaction between the CaSR and calcium and its protective action against CRC. A randomised clinical trial found that dietary calcium supplementation increased the expression of the CaSR in the colonic mucosa<sup>[92]</sup>. In a meta-analysis, Yang *et al.*<sup>[93]</sup> showed that while dietary calcium reduced the risk of developing CaSR positive tumours, the risk for CaSR negative ones remained unchanged, suggesting that dietary calcium exerts its anti-tumourigenic properties *via* CaSR. A recent study demonstrated that CaSR expression in the tumours correlated with a reduced risk of mortality, indicating that CaSR expression might be a biomarker for positive prognosis<sup>[94]</sup>.

### CaSR localisation in the intestine

A common agreement on the pattern of CaSR localization in the intestine is still missing. Whitfield suggested that the  $\text{Ca}^{2+}$  concentration is unevenly distributed along the colonic crypts, with low levels found at the bottom of the crypts and higher levels at the top. In this way,  $\text{Ca}^{2+}$  could exert its pro-differentiating and anti-proliferative effects only in the upper part of the crypts where the post-differentiated mature colonocytes are localised. CaSR activation would follow this concentration gradient along the crypts. Stronger activation of the CaSR at the top and weaker activation at the bottom could thus provide a physiological rationale for why the CaSR would inhibit proliferation on the differentiated top but allow proliferation at the rapidly dividing bottom of the

crypts<sup>[95]</sup>. It was also suggested that this  $\text{Ca}^{2+}$  gradient influences CaSR expression itself, in addition to the receptor's activation<sup>[46]</sup>. This theory is supported by the studies of Chakrabarty *et al.*<sup>[96]</sup>, who have found CaSR protein to be expressed only in the upper half of the crypts of human colon cancer biopsies. However, the actual expression pattern of the CaSR in the colon is still under debate. Contrary to the findings by Chakrabarty *et al.*<sup>[96]</sup>, Sheinin *et al.*<sup>[97]</sup> have found CaSR expression only in the enteroendocrine cells of human colonic mucosa<sup>[97]</sup>, whereas Cheng *et al.*<sup>[8]</sup> have found the CaSR in the enteric nervous system and in the apical and basolateral side of the crypts of rat colons<sup>[8,98]</sup>. Further studies are therefore required to determine accurately the location of the CaSR in the colon and whether this expression pattern is dependent on factors like diet, age, *etc.*

We know that CaSR expression is lost in tumour cells. While it is still found in pre-neoplastic lesions, expression of the CaSR is lost in poorly differentiated tumours<sup>[29,96,99,100]</sup>. However, whether this loss is cause or effect of the tumourigenesis is still unknown.

### CaSR down-regulation in CRC

Epigenetic aberrancies play a major role in tumour malignancy in general and thereby also in colorectal cancer<sup>[101]</sup>. CaSR expression is affected by repressive epigenetic marks in malignant colorectal lesions. The promoter region of the CaSR contains a large CpG island which is highly methylated in colorectal tumours. CaSR expression could be partially restored in colorectal cancer cell lines by the administration of 5-aza-2'-deoxycytidine, an inhibitor of DNA methylation. This effect was further enhanced with the addition of histone deacetylase inhibitors, suggesting that in the CaSR promoter regions the acetylation of histones is reduced and, therefore, the chromatin has a less permissive structure that hinders the recruitment of the transcription machinery<sup>[29,102]</sup>. The level of CaSR methylation increases from hyperplastic polyps and adenomas to lymph node metastases in parallel with the reduction of the receptor's expression<sup>[102]</sup>. However, this is not a general mechanism, as in parathyroid tumours no hypermethylation of the CaSR locus was found<sup>[64,103]</sup>.

Non-coding RNAs, such as miRNAs, also regulate CaSR expression in colorectal tumours. Different studies found that miR-21, miR-135a, miR-135b, miR-145, miR-146b and miR-503 inhibited CaSR expression in CRC cell lines and therefore constitute potential targets for restoring CaSR mRNA level<sup>[61,104,105]</sup>.

So far, no CaSR mutations have been found that would promote tumour development in the intestine<sup>[48]</sup>, although several SNPs (*e.g.*, Q1011E, A986S, R990G) might increase colorectal cancer susceptibility although their contribution is controversial<sup>[106-109]</sup>.

It is important to fully understand the molecular mechanisms that drive CaSR loss during colorectal carcinogenesis and whether this loss could be reverted or prevented and whether such an action would be

beneficial for patient prognosis, pointing towards the CaSR as potential therapeutic target for a novel anti-CRC therapy or prevention.

### Evidence and molecular pathways for the anti-tumourigenic actions of the CaSR

Mouse models of systemic CaSR knock-out are not viable or die shortly after birth due to severe hyperparathyroidism and hypercalcemia<sup>[110]</sup>. However knocking out PTH rescues the lethal CaSR<sup>-/-</sup> phenotype in the PTH double knock-out (PTH<sup>-/-</sup> CaSR<sup>-/-</sup>) mouse model<sup>[111]</sup>. The colonic mucosa of the PTH<sup>-/-</sup> CaSR<sup>-/-</sup> mice as well as that of the intestinal epithelium-specific CaSR knock-out mouse model show signs of hyperproliferation. These mice develop pre-malignant intestinal lesions and are highly susceptible to the carcinogen azoxymethane (AOM)<sup>[112]</sup>. The intestines of these mice are often inflamed and express pro-inflammatory markers. Furthermore, PTH<sup>-/-</sup> CaSR<sup>-/-</sup> mice are highly sensitive to DSS induced inflammation as well, suggesting a possible role of the CaSR as an anti-inflammatory factor<sup>[42,112]</sup>.

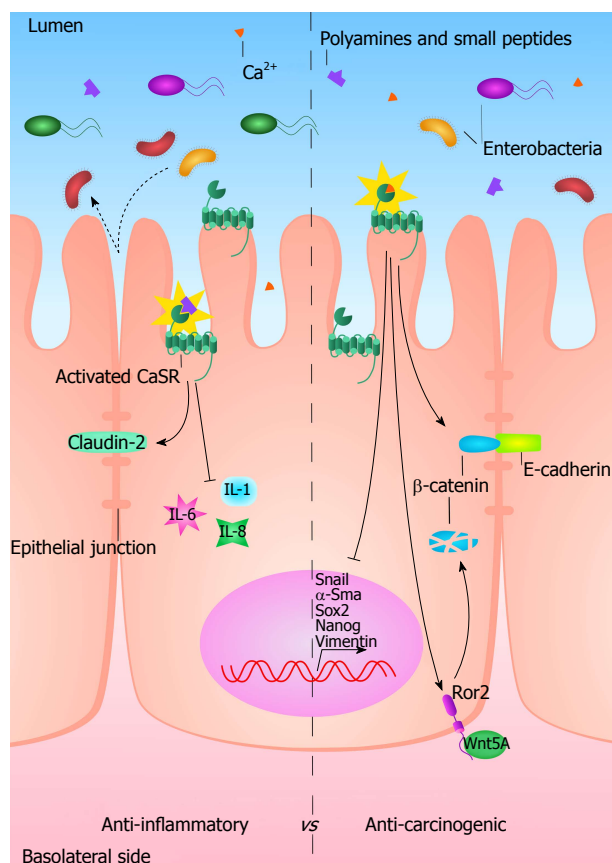
Overexpression of the exogenous CaSR in colon cancer cell lines induced cellular differentiation and apoptosis, and inhibited proliferation and invasion capacity in these transfected cells. Presence of the CaSR repressed expression of stem cells markers, re-established the expression of E-cadherin and inhibited epithelial to mesenchymal transition, a process exploited by cancer cells to form metastases<sup>[47,59]</sup>.

$\text{Ca}^{2+}$  exerts its anti-tumourigenic function not only by binding and precipitating toxic agents such as secondary bile acids and fatty acids but also by modulating different cellular mechanisms such as proliferation, differentiation and apoptosis, potentially *via* the CaSR<sup>[3,100,113,114]</sup>. The mechanism involves inhibition of c-myc, upregulation of E-cadherin and inhibition of the canonical wnt-signalling pathway<sup>[99,100,115]</sup>. A recent study reported that  $\text{Ca}^{2+}$  inhibited the expression of replication-licensing factors in a CaSR dependent manner<sup>[60]</sup>.

Both colorectal cancer cell lines and CaSR-deficient mice show that loss of the CaSR causes a higher recruitment of  $\beta$ -catenin into the nucleus, thus sustaining a proliferative Wnt pathway<sup>[59,112,116]</sup>. However, some studies have discovered that the CaSR is able to activate the non-canonical Wnt pathway involving the interaction between Wnt5a and its receptor, Ror2 (receptor tyrosine kinase-like orphan receptor 2). Wnt5a/Ror2 counteracts the proliferative signalling of Wnt/ $\beta$ -catenin, recruiting the ubiquitin ligase Siah2, which, in turn, degrades  $\beta$ -catenin. In myofibroblasts, CaSR activation induces the secretion of Wnt5a, while, in colonic epithelia CaSR increases the expression of Ror2<sup>[62]</sup>. Thus, the CaSR might stimulate the Wnt5a/Ror2 paracrine pathway which inhibits colonic proliferation, interfering with Wnt/ $\beta$ -catenin, and seems to promote the expression of colonic differentiation markers such as sucrase-isomaltase, caudal type homeobox 2 and villin<sup>[62,117,118]</sup>.

CaSR pathways could potentially interact with many cellular processes in preventing or counteracting





**Figure 2** Protective function of the calcium-sensing receptor against inflammation and carcinogenesis in the colon. Left: The CaSR promotes intestinal barrier integrity, potentially by promoting claudin-2 expression, and inhibits the expression of pro-inflammatory cytokines, thus preventing inflammation. Right: The CaSR exerts an anti-tumorigenic effect by counteracting the mitogenic Wnt pathway, preventing  $\beta$ -catenin translocation into the nucleus, which is either sequestered by E-cadherin at the cell junctions or it is degraded by the non-canonical Wnt signalling (Ror2-Wnt5A) and inhibits the expression of mesenchymal and stem cells markers. IL: Interleukin; CaSR: Calcium-sensing receptor.

tumour development and progression. In this context, the existence of a cross talk between the CaSR and the vitamin D system has been suggested. It seems that both pathways converge in the modulation of the Wnt signalling to control colonocyte proliferation. Moreover, vitamin D seems to regulate CaSR transcription<sup>[119,120]</sup> through regulatory elements present in the CaSR promoter, which are recognised by the transcription factor vitamin D receptor. Indeed, a high vitamin D (2500 IU/kg) diet over 5 weeks more than doubled the expression of the CaSR in the colon mucosa of mice<sup>[121,122]</sup>.

As of yet, a detailed description of CaSR signalling in the intestine is still missing. Given the fact that the CaSR is able to sense not only  $\text{Ca}^{2+}$ , but also polyamines and amino acids, which are highly abundant in the intestinal lumen through the food, and that ligand biased signalling is a known feature of the CaSR<sup>[3]</sup>, it is possible that the CaSR could activate different down-stream signals depending on these specific ligands also in the colon. Potential mechanisms by which the CaSR could affect

inflammation and CRC are summed up in Figure 2 but a detailed map of the molecular pathways that the CaSR activates in the gut is still missing. This would allow researchers to discover potential therapeutic targets for counteracting intestinal tumourigenesis.

## FUTURE PERSPECTIVES - THE CaSR AS A DRUGGABLE TARGET IN THE COLON

The CaSR is considered to prevent or counteract intestinal carcinogenesis and inflammation. Thus, the CaSR might constitute a promising therapeutic target for the treatment of colorectal cancer and of inflammatory bowel diseases. Dietary  $\text{Ca}^{2+}$  supplementation reduces the risk for developing colorectal cancer and studies have shown the beneficial effects of CaSR agonists, such as dipeptides, polyamines for preventing colonic inflammation and cancer. As chronic inflammation is a risk factor for colorectal cancer, the CaSR might actually be a link that connects the beneficial effect of  $\text{Ca}^{2+}$  in preventing both inflammation and cancer in the colon. Indeed, these roles of the CaSR indicate that activating the CaSR, or in the case of CRC also restoring CaSR expression - or preventing its loss - might be an important way for treating or preventing colonic inflammation, CRC, and, especially, CAC. However, a direct pharmacological intervention study targeting the CaSR in colonic inflammation or colorectal cancer is still missing.

Further research will be required for finding and evaluating means to restore or prevent the loss of the expression of the CaSR during carcinogenesis. One such mean could be the use of pharmacological CaSR activators, the calcimimetics. In addition to their action as allosteric agonists of the CaSR, calcimimetics also act as so called "pharmacochaperones" for the CaSR. They stabilise the expression of the CaSR, preventing the receptor's degradation. At the same time, they increase trafficking of the CaSR from its intracellular reservoirs into the cell membrane<sup>[123,124]</sup>. As of yet, there are no data for the efficacy of calcimimetics for the prevention / treatment of CRC or CAC.

As chronic inflammation is posing a high risk for developing CAC, preventative measures should be administrable over long periods of time and should therefore ideally elicit few or no systemic side effects. Recently a novel calcimimetic, GSK3004774, which is non-resorbable and thus has gut restricted effects, has been published<sup>[125]</sup>. This compound could be useful for testing whether locally acting calcimimetics can elicit a preventive effect against intestinal inflammation, CRC and CAC without affecting systemic calcium homeostasis.

Known side effects of the FDA-approved calcimimetic cinacalcet treatment include hypocalcaemia and, notably, nausea<sup>[126]</sup>. Whether these gastrointestinal tract-related side effects are elicited *via* the systemic actions of the drug or a direct effect of the drug on the gastrointestinal

organs is unclear. In addition, calcimimetics have been shown to actually enhance inflammation in other epithelial tissues, *e.g.*, the lung, while calcilytics, antagonists of the CaSR, ameliorated the inflammation. In this context, the CaSR also promoted the activation of the immune system and showed a general pro-inflammatory action<sup>[5]</sup>. Whether these *in vivo* effects - in the complex context of immune-cells, inflamed tissue and cytokines - are tissue specific or related to a ubiquitous activation of CaSR-bearing lymphocytes is unclear. Taken together, these considerations do not allow a definite conclusion for a potential treatment of colonic inflammation or cancer with pharmacological CaSR modulators alone or in combination with conventional or targeted chemotherapies. Extensive future studies will be required to satisfactorily answer all these questions.

## CONCLUSION

The CaSR emerges as a direct player in colonic inflammation and cancer development. Current evidence suggests that activation of the CaSR reduces the risk for both diseases, the strongest evidence being that dietary  $\text{Ca}^{2+}$  reduces the risk for CRC and that this effect is apparently mediated by the CaSR while expression of the CaSR is lost during tumourigenesis and progression of CRC. Making direct use of the CaSR as a drug target to reduce or prevent colonic inflammation and at the same time prevent colonic tumourigenesis seems a promising strategy, especially for CAC, where a dietary or pharmacological intervention could hit two birds with one stone, as it were. Future studies will be needed to address where exactly the receptor is expressed in the colonic microenvironment, which signalling pathways are mediated by the CaSR in the settings of inflammation and cancer *in vivo*, and whether these actions of the CaSR can be exploited for therapy and prevention.

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## Production of extracellular lysophosphatidic acid in the regulation of adipocyte functions and liver fibrosis

Fang Yang, Guo-Xun Chen

Fang Yang, School of Laboratory Medicine, Hubei University of Chinese Medicine, Wuhan 430065, Hubei Province, China

Guo-Xun Chen, Department of Nutrition, University of Tennessee at Knoxville, Knoxville, TN 37996, United States

ORCID number: Fang Yang (0000-0002-7092-2532); Guo-Xun Chen (0000-0001-6226-4050).

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**Correspondence to:** Guo-Xun Chen, MD, PhD, Associate Professor, Research Scientist, Department of Nutrition, University of Tennessee at Knoxville, Room 229, Jessie Harris Building, 1215 West Cumberland Avenue, Knoxville, TN 37996, United States. [gchen6@utk.edu](mailto:gchen6@utk.edu)  
Telephone: +1-865-9746254  
Fax: +1-865-9743491

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### Abstract

Lysophosphatidic acid (LPA), a glycerophospholipid, consists of a glycerol backbone connected to a phosphate head group and an acyl chain linked to sn-1 or sn-2 position. In the circulation, LPA is in sub-millimolar range and mainly derived from hydrolysis of lysophosphatidylcholine, a process mediated by lysophospholipase D activity in proteins such as autotaxin (ATX). Intracellular and extracellular LPAs act as bioactive lipid mediators with diverse functions in almost every mammalian cell type. The binding of LPA to its receptors LPA<sub>1-6</sub> activates multiple cellular processes such as migration, proliferation and survival. The production of LPA and activation of LPA receptor signaling pathways in the events of physiology and pathophysiology have attracted the interest of researchers. Results from studies using transgenic and gene knockout animals with alterations of ATX and LPA receptors genes, have revealed the roles of LPA signaling pathways in metabolic active tissues and organs. The present review was aimed to summarize recent progresses in the studies of extracellular and intracellular LPA production pathways. This includes the functional, structural and biochemical properties of ATX and LPA receptors. The potential roles of LPA production and LPA receptor signaling pathways in obesity, insulin resistance and liver fibrosis are also discussed.

**Key words:** Autotaxin; Lysophosphatidic acid receptors; Obesity; Lysophosphatidic acid; Insulin resistance; Liver fibrosis

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**Core tip:** Lysophosphatidic acid (LPA) is mainly derived from hydrolysis of lysophosphatidylcholine, a process mediated by lysophospholipase D activity in proteins such as autotaxin (ATX). The binding of LPA to its receptors LPA<sub>1-6</sub> activates multiple cellular signaling pathways and leads to changes. Studies using genetically modified animals have begun to reveal the roles of LPA pathways in metabolic active tissues and organs. The present review summarized recent progresses in the studies of extracellular and intracellular LPA production pathways; the functions, structural and biochemical properties of ATX and LPA receptors. Furthermore, the potential roles of LPA production and LPA receptor signaling pathways in obesity, insulin resistance and liver fibrosis are discussed.

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## INTRODUCTION

Lysophosphatidic acid (1- or 2-acyl-sn-glycerol 3-phosphate/radyl-glycerol-phosphate, LPA) is one type of water-soluble glycerophospholipid with molecular mass about 430-480 Da. All LPA molecules contain a glycerol backbone linked to a phosphate head group at sn-3 position and an acyl chain esterified to sn-1 or sn-2 position. Due to variation of the fatty acyl chain, LPA molecules are in different forms and derived from multiple sources, such as membrane lipids<sup>[1]</sup>. The LPA molecules produced extracellularly exert a variety of physiological responses after binding to their receptors as shown in Figure 1.

LPA was first identified as an active ingredient of Darmstoff by Vogt in 1957<sup>[2,3]</sup>. The term Darmstoff was used to describe a smooth-muscle-stimulating substance which was first observed with bath fluid of isolated intestine preparations<sup>[4]</sup>. The substance was acidic and soluble in many organic solvents, properties that distinguish Darmstoff from amines and polypeptides<sup>[5]</sup>. Results of acidic hydrolysis and paper chromatography showed that the smooth-muscle-stimulating activity of Darmstoff was due to a mixture of acidic phospholipids (PLs), one of which is an acetal phosphatidic acid<sup>[2,3]</sup>. In the 1960s, studies on smooth muscle and blood pressure suggested that LPA had biological activities<sup>[6,7]</sup>. Later on, various molecules of LPA species were isolated and identified from soy beans. It was shown that intravenous injection of LPA from crude soybean lecithin caused hypertension in rats and guinea pigs, but hypotension in cats and rabbits<sup>[8]</sup>. This raised intriguing questions regarding the activation mechanism of this lipid specie. Since then, the myriad

biological effects of LPA have drawn attention of biomedical scientists.

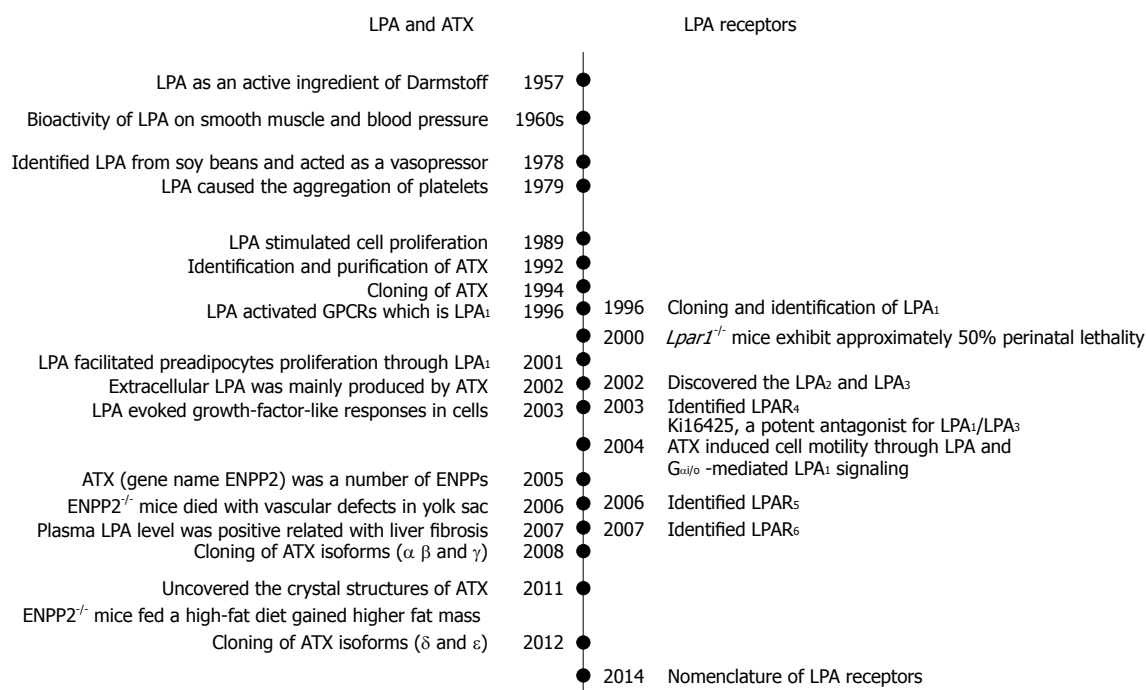
Subsequently, LPA in incubated serum at 36 °C for 18-24 h was shown to cause aggregation of feline and human platelets<sup>[9]</sup>. Whether LPA acts through its detergent-like physical property or its interaction with a specific receptor remained a critical question. Later, LPA was shown to stimulate cell proliferation in a pertussis toxin-sensitive manner<sup>[10]</sup>. This finding suggested that LPA acts through G protein-coupled receptor (GPCR). This information led to the cloning and identification of a GPCR, which is now known as LPA receptor 1 (LPA<sub>1</sub>)<sup>[11]</sup>. It is known now that as a bioactive lipid mediator, LPA activates at least 6 specific GPCRs, named as LPA<sub>1-6</sub>. These GPCRs are coupled with several G $\alpha$  proteins such as G $\alpha_{12/13}$ , G $\alpha_{q/11}$ , G $\alpha_{i/o}$ , and G $\alpha_s$ . The binding of LPA to these receptors stimulates the activations of small GTPases, Ras, Rho, and Rac, and induces downstream actions<sup>[12]</sup>.

The existence of extracellular LPA indicates its production outside a cell. Since autotaxin (ATX) was first identified from human plasma and found to be a lysophosphatidic acid-producing enzyme in 2002<sup>[13]</sup>, the ATX-LPA receptor signaling pathway has been implicated in a variety of disease processes including the vascular and neural development, hair follicle development, tumor progression, lymphocyte trafficking, bone development, pulmonary fibrosis, fat mass regulation, cholestatic pruritus, neuropathic pain, embryo implantation, obesity and glucose homeostasis, spermatogenesis, fetal hydrocephalus, chronic inflammation, cellular proliferation, and smooth muscle contraction during development<sup>[14-18]</sup>. Both ATX and LPA have attracted the interest of researchers in an effort to understand their roles in pathophysiology and to develop new agents to treat above-mentioned pathological conditions.

## EXTRACELLULAR AND INTRACELLULAR PRODUCTION AND DEGRADATION OF LPA

LPA and its common precursor lysophosphatidylcholine (LPC) can be found both extracellular and intracellular as signaling mediators and membrane components, respectively. Structurally, LPA is an acyl group esterified to the sn-1 or sn-2 position of the glycerol backbone. Due to the differences of acyl chain length, saturation and backbone position, various LPA chemical forms can be found in tissues and cells. Extracellular LPA is thought to mediate bioactive effects through LPA receptors<sup>[19]</sup>. Intracellular LPA is an important intermediate for the *de novo* biosynthesis of complex glycerolipids, including mono-, di-, and triglycerides, as well as PLs<sup>[20]</sup>. In addition, it has been thought that LPA can function as a ligand for transcription factor peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ )<sup>[21]</sup>. This indicates that LPA may play important roles in the regulation of gene expression.





**Figure 1** Chronological events related the identifications of lysophosphatidic acid, ecto-nucleotide pyrophosphatase/phosphodiesterase/autotaxin and lysophosphatidic acid receptors. On the left side, it shows the events associated with the identifications of LPA molecules and ATX for its production. On the right side, it shows the cloning events of LPAR<sub>1-6</sub>. LPA: Lysophosphatidic acid; ATX: Autotaxin; LPA<sub>1-6</sub>: Lysophosphatidic acid receptor 1-6; ENPP2: Ectonucleotide pyrophosphatase/phosphodiesterase family member 2.

### Pathways for LPA production

As shown in Figure 2, there are five major pathways for LPA production, (1) the lysophospholipids-ATX (LPLs-ATX) pathway, (2) the phosphatidic acid - phospholipase A<sub>1</sub> or A<sub>2</sub> (PA-PLA<sub>1</sub>/PLA<sub>2</sub>) pathway, (3) the *de novo* glycerophosphate acyltransferase (GPAT) synthesis pathway, (4) the monoacylglycerol kinase (MAGK) pathway, and (5) the oxidative modification of low-density lipoprotein (LDL) pathway. Despite recent advances in the identification of the enzymes responsible for LPA production, the regulation of these enzymes still remains obscure.

#### LPLs-ATX pathway

In the first pathway, LPLs generated from PLs by PLA<sub>1</sub> or PLA<sub>2</sub> are converted to LPA by a plasma enzyme ATX<sup>[22,13]</sup>, which we will describe in later part of this article. A major source of extracellular LPA is LPC, other LPLs such as lysophosphatidylserine and lysophosphatidylethanolamine can also be enzymatically processed to produce LPA. This pathway accounts for the majority of circulating LPA.

#### PA-PLA<sub>1</sub>/PLA<sub>2</sub> pathway

LPA is also produced intracellularly as an intermediate for the synthesis of other glycerolipids<sup>[20]</sup>. LPA can be produced enzymatically from intracellular organelles such as mitochondria and endoplasmic reticulum. Phosphatidic acid (PA) is first generated from PLs or diacylglycerol by phospholipase D enzymes (PLD<sub>1</sub> and PLD<sub>2</sub>) and diacylglycerol kinase (DGK) activities,

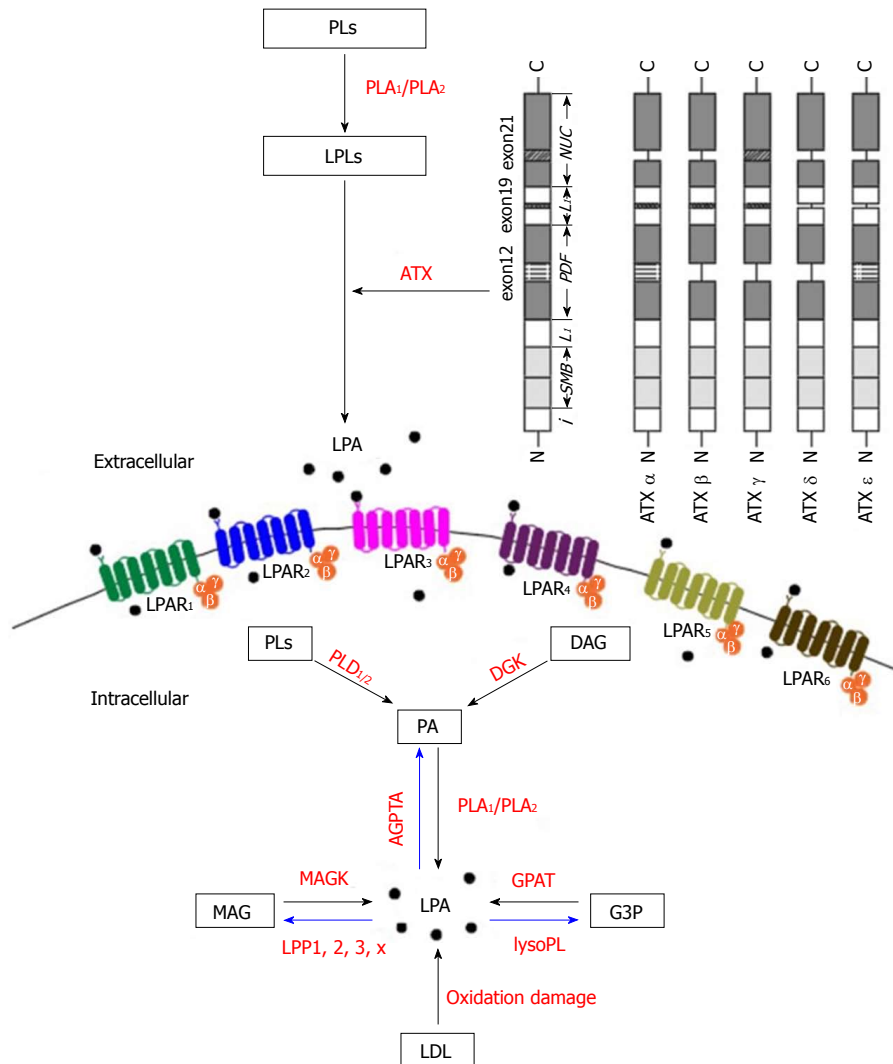
respectively. Then, one acyl group is removed from the sn-1 position by PLA<sub>1</sub> or at the sn-2 position by PLA<sub>2</sub> enzymes to generate LPA. This pathway may be more important in specific tissues with expression of DGK such as the brain and skin<sup>[23]</sup>.

#### De novo GPAT synthesis pathway

GPATs catalyze the first step in glycerolipid synthesis, *i.e.*, the conversion of glycerol-3-phosphate (G3P) to LPA by the transfer of fatty acids from acyl-CoA. Since GPAT exhibits the lowest specific activity of enzymes in the *de novo* triacylglycerol (TAG) and PLs synthesis pathways, it has been considered to be the rate limiting enzyme for them<sup>[24]</sup>. Many studies have been published on the regulation of TAG synthesis and its relevance to obesity and insulin resistance. GPAT activity in mitochondria was shown to be regulated by fatty acid-binding protein (FABP)<sup>[25,26]</sup>. It has been shown that mitochondrial GPAT activity was inhibited by LPA. FABP reversed the inhibition of LPA through the binding and extracting LPA from the mitochondrial outer membrane. The extracted LPA was converted to PA by microsomes, where acylglycerophosphate acyltransferases (AGPATs) are located<sup>[25,26]</sup>. These results suggested that FABP regulated the *de novo* synthesis of PA through the stimulation of mitochondrial GPAT and transport of LPA from mitochondria to microsomes.

#### MAGK pathway

Lipid phosphate phosphatases (LPPs) are also involved in the LPA turnover. LPPs can be found extracellularly



**Figure 2 Biochemical pathways of lysophosphatidic acid synthesis and degradation.** LPA can be produced extracellularly and intracellularly as signaling mediators and membrane components, respectively. There are five major pathways for LPA production, (1) the lysophospholipids-ATX (LPLs-ATX) pathway, (2) the phosphatidic acid - phospholipase A<sub>1</sub> or A<sub>2</sub> (PA-PLA<sub>1</sub>/PLA<sub>2</sub>) pathway, (3) the *de novo* glycerophosphate acyltransferase (GPAT) synthesis pathway, (4) the monoacylglycerol kinase (MAGK) pathway, and (5) the oxidative modification of low-density lipoprotein (LDL) pathway. In the upper right corner of the figure, there are catalytically active isoforms (ATX $\alpha$ , ATX $\beta$ , ATX $\gamma$ , ATX $\delta$  and ATX $\epsilon$ ), which are expressed in different tissues. PLs: Phospholipids; PLA<sub>1</sub>/PLA<sub>2</sub>: Phospholipase A<sub>1/2</sub>; LPLs: Lysophospholipids; ATX: Autotaxin; ATX $\alpha$ - $\delta$ : Protein structure scheme of the domains of ATX; LPA: Lysophosphatidic acid; DAG: Diacylglycerol; DGK: Diacylglycerol kinase; PLD<sub>1/2</sub>: Phospholipase D<sub>1/2</sub>; PA: Phosphatidic acid; AGPAT: Acylglycerophosphate acyltransferase; MAG: Monoacylglycerol; MAGK: Monoacylglycerol kinase; LPP: Lipid phosphate phosphatase; G3P: Glycerol-3-phosphate; lyso PL: Lysophospholipase; GPAT: Glycerophosphate acyltransferase; LDL: Low-density lipoprotein; i: Intramembrane domain; SMB: N-terminal somatomedin B-like domains; L<sub>1</sub>: L<sub>1</sub> linker region; PDF: Phosphodiesterase domain; L<sub>2</sub>: L<sub>2</sub> linker region; NUC: C-terminal nuclease-like domain; LPA<sub>1-6</sub>: Lysophosphatidic acid receptor 1-6.

or intracellularly in endoplasmic reticulum or Golgi, where they dephosphorylate LPA, which leads to the formation of monoacylglycerol (MAG)<sup>[27]</sup>. MAG may then be phosphorylated by MAGK and thus participate in another round of LPA signaling<sup>[20]</sup>. Thus, the production of LPA is regulated by the availability of precursors as well as the expression of catalytic enzymes.

### Oxidative modification of LDL

LPA was found as an active molecule on oxidized and modified LDL, in where it may contribute to platelet activation, endothelial cell stress-fiber and gap formation<sup>[28,29]</sup>. LPAs on these lipoproteins activate platelets through G-protein coupled LPA receptors and

a Rho/Rho kinase signaling pathway, which leads to platelet shape change and subsequent aggregation. The biologically active LPA-like products generated by this non-enzymatic oxidation co-migrated with an authentic LPA standard in thin layer chromatography. LPA was found to be accumulated in atherosclerotic plaques, which might act to activate platelets. The level of LPA is very high in the human carotid atherosclerotic lesion, suggesting the roles in thrombogenesis and rupture<sup>[28,29]</sup>. An alternative explanation to the generation of LPA from oxidized LDL is that ATX might be activated. The acyl/alkyl composition, the precursor of LPA and the mechanism responsible for LPA generation in the oxidized LDL remain to be addressed in the future.

### Pathways for the degradation of LPA

There are three major pathways that degrade LPA as shown in Figure 2. The first is the removal of phosphate to form MAG by LPPs<sup>[30]</sup>. LPA has a half-life of 3 min when it is added to cells expressing LPP<sup>[31]</sup>. Four isoforms of LPP have been cloned and characterized in mammals, LPP1/PAP-2 $\alpha$ /PAP-2 $\alpha$ 1<sup>[32]</sup>, LPP1 $\alpha$ /PAP-2 $\alpha$ 2<sup>[33]</sup>, LPP2/PAP-2 $\gamma$ /PAP-2 $\alpha$ <sup>[34]</sup> and LPP3/PAP-2 $\beta$ /PAP-2 $\beta$ <sup>[32]</sup>. The second LPA degradation pathway involves the action of AGPAT enzymes, also known as lysophosphatidic acid acyltransferase. These microsomal enzymes catalyze the transfer of an acyl group from acyl-CoA to LPA to form PA. Proteins with AGPAT activity include a family of transmembrane enzymes<sup>[35]</sup>, and membrane associated proteins involved in membrane fission such as endophilin<sup>[36]</sup> and C-terminal-binding protein/brefeldin A-ADP ribosylated substrate<sup>[37]</sup>. The third pathway for LPA degradation involves the hydrolysis of the acyl group from the G3P head group by the action of lysophospholipases. The majority of characterized lysophospholipases act on LPC<sup>[38]</sup>.

### ECTO-NUCLEOTIDE PYROPHOSPHATASE/PHOSPHODIESTERASES

The ecto-nucleotide pyrophosphatase/phosphodiesterase (ENPP) family contains seven members with structurally similar catalytic domains that hydrolyze phosphodiester bonds in various substrates, including nucleoside triphosphates, LPLs, and choline phosphate esters<sup>[39,40]</sup>. ATX, or ENPP2, is the best-characterized member of ENPP family. ENPPs are defined by their ability to hydrolyze phosphodiester bonds of various nucleotides *in vitro*<sup>[41-43]</sup>. ATX/ENPP2 was originally identified as a tumor cell-motility-stimulating factor from the conditional medium of A2058 human melanoma cells<sup>[44]</sup>. Since the addition of pertussis toxin reduced cellular motility, ATX's effects were thought to involve G<sub>i/o</sub>-mediated signaling<sup>[39,44]</sup>. ATX can be secreted as a 100 kDa glycoprotein. It is produced by multiple tissues including adipose tissue<sup>[45,46]</sup>. It is believed that the circulated ATX/ENPP2 is degraded by the liver<sup>[47]</sup>. Extracellular LPA was found to be present in sub-micromolar ranges. The responsible enzyme was identified to be ATX<sup>[13,22]</sup>. ATX-mediated autocrine signaling induces cell motility through LPA production and G<sub>i/o</sub>-mediated LPA receptor signaling<sup>[48]</sup>.

From both a structural and evolutionary point of view, ENPP family members have been categorized into two subgroups, ENPP1-3 and ENPP4-7<sup>[39]</sup>. ENPP1-3 all have two N-terminal somatomedin B-like (SMB) domains, a central phosphodiesterase (PDE) domain and a C-terminal nuclease (NUC)-like domain as shown in Figure 2. ENPP4-7 only have similarity in the PDE domain. The crystal structures of mouse<sup>[49]</sup> and rat<sup>[50]</sup> ATX show loops on both sides of the catalytic domain, which may help to determine the binding specificity. The SMB and PDE domains are connected by the first loop (L<sub>1</sub> linker region), whereas the PDE and NUC domains are

connected by the second loop (L<sub>2</sub> linker region)<sup>[49]</sup>.

The secreted ATX is a constitutively active glycoprotein with a N-terminal signal peptide sequence containing a furin cleavage site<sup>[46]</sup>. The other ENPPs are transmembrane or anchored proteins. In addition to its pyrophosphatase/phosphodiesterase activities, ATX has lysophospholipase D (lysoPLD) activity. The N-terminal signal peptide of the ATX precursor is removed first, and then, the remaining part is cleaved by proprotein convertases before the active ATX with lysoPLD activity is released into the extracellular environment, which converts LPC into LPA and choline<sup>[47]</sup>. The structure of PDE domain has a lipid binding pocket and a nearby tunnel allowing entry of substrates and release of products<sup>[51]</sup>. The NUC domain has been thought to maintain the rigidity of the PDE domain, and the two N-terminal SMB domains mediate binding of ATX to integrin<sup>[52]</sup>. This binding brings ATX to the cell membrane, which allows the production of LPA in a location close to its receptors<sup>[51,53,54]</sup>.

The structures of ATX in complex with diverse LPAs show distinct conformations after different acyl chains occupy the binding pocket<sup>[49]</sup>. LPAs with saturated chains bind in the hydrophobic pocket in a more elongated fashion, whereas LPAs with unsaturated chains have a bent conformation due to the presence of carbon-carbon double bond(s). For LPA (22:6), the acyl chain shows a U-shaped conformation in the binding pocket<sup>[49]</sup>. ATX prefers LPC species with shorter and unsaturated acyl chain as substrates, and the rank order is 14:0 > 16:0 > 18:3 > 18:1 > 18:0. All these show that ATX is able to hydrolyze LPCs with different lengths and saturations of acyl chains to produce the corresponding LPAs.

The cDNA of ATX/ENPP2 was cloned in 1994<sup>[55]</sup>. After that, its homology with phosphodiesterases was revealed, and the cloning and tissue distribution of the three human and mouse isoforms ( $\alpha$ ,  $\beta$  and  $\gamma$ ) were determined in 2008<sup>[50]</sup>. Two more isoforms ( $\delta$  and  $\epsilon$ ) were identified in 2012<sup>[56]</sup>. The ATX gene is located on mouse chromosome 15 and on human chromosome 8. The human and mouse ATX gene structures are conserved<sup>[50]</sup>. The mouse ATX gene spans more than 80 kb and contains at least 27 exons. The three splicing sites in exons 12, 19 and 21 can theoretically result in eight isoforms, in which five were detected. These isoforms are catalytically active (ATX  $\alpha$ - $\delta$ ) and expressed in different tissues. They are ATX $\alpha$  (ATXm), ATX $\beta$  (ATXt), ATX $\gamma$  (PD-I $\alpha$ )<sup>[50]</sup>, ATX $\delta$  and ATX $\epsilon$ <sup>[56]</sup>. ATX $\beta$  and ATX $\delta$ , which are the most and second most abundant isoforms, respectively, share similar biochemical characteristics (Figure 2). Houben *et al.*<sup>[53]</sup> characterized that a 52-residue polybasic insertion corresponding to exon 12 in ATX $\alpha$  isoform confers specific binding to heparan sulfate proteoglycans thereby targeting LPA production to the plasma membrane. This is another potential mechanism for localizing ATX $\alpha$  to cell membranes and for LPA production in close proximity to LPA receptors. Exon 12 encodes a 52-amino acid insertion of the mouse ATX $\alpha$  and ATX $\epsilon$

isoforms (amino acids 324-375), whereas exon 21 encodes an additional 25-amino acid of the murine ATX $\gamma$  isoform (amino acids 593-617). Novel isoforms ATX $\delta$  and ATX $\epsilon$  have a 4-amino acid deletion on exon 19. This complex way of exon arrangement has been maintained through evolution. Human ATX exhibit 93% sequence identity with rodent ATX while all important residues are highly conserved<sup>[49]</sup>.

ATX has a broad profile of tissue expression, with relatively high levels in the blood, brain, kidney, and lymphoid organs<sup>[57-59]</sup>. Secretion of ATX leads to high concentration in cerebrospinal fluid and in the endothelial venules of lymphoid tissues<sup>[60-62]</sup>. The cellular sources of plasma ATX are incompletely understood. Nevertheless, adipocytes may be a source<sup>[63,64]</sup>. ATX is also stored in platelets and released during their activation<sup>[65,66]</sup>. Circulating ATX is rapidly taken up by the scavenger receptors of liver sinusoidal endothelial cells, and then degraded by the liver<sup>[46]</sup>. Thus, like insulin, ATX is largely removed from the circulation through first passage by the liver. For the ATX isoforms, high expression levels of ATX $\beta$  and ATX $\gamma$  mRNA were detected in peripheral tissues and the brain, whereas ATX $\alpha$  was shown the lowest expression level in both the central nervous system and peripheral tissues among the three isoforms in human. In mice, ATX $\beta$  is widely expressed in the brain and peripheral tissues, and ATX $\gamma$  and ATX $\alpha$  showed little variation in their distribution<sup>[50]</sup>. Human brain and retina showed relatively higher expression level of ATX $\alpha$  than that of ATX $\beta$  and ATX $\gamma$ , whereas the expression levels of ATX $\delta$  and ATX $\epsilon$  in the small intestine and spleen are higher than that in other tissues<sup>[56]</sup>.

LPA has been quantified in a variety of species, tissues, and fluids, including neural tissue, cerebrospinal fluid, fertilized hen white, seminal fluid, tears, plasma, serum, urine, saliva, and aqueous humor<sup>[67-69]</sup>. The formation of LPA species depends on the precursor PLs, which can vary by acyl chain length and degree of saturation. The term LPA most often refers to 18:1 oleoyl-LPA (1-acyl-2-hydroxy-sn-glycero-3-phosphate), as it is the most commonly one. Other chemical forms of LPA can be observed in various biological systems that have concentrations ranging from low nanomolar to micromolar levels<sup>[67,70]</sup>. LPA concentrations in human and rat blood can range from 0.1  $\mu\text{mol/L}$  in plasma and up to 10  $\mu\text{mol/L}$  in serum, which is well over the apparent nanomolar kDa of LPA $_{1-6}$ <sup>[71-74]</sup>. The LPA molecules containing 18:2, 20:4, 16:1, 16:0, and 18:1 acyl chains are particularly abundant in plasma<sup>[75-77]</sup>. Current methods to detect LPA include indirect enzymatic assays<sup>[73]</sup>, TLC-GC, LC-MS, and LC-MS/MS<sup>[78-80]</sup>.

## LPA RECEPTORS-MEDIATED LPA SIGNALING

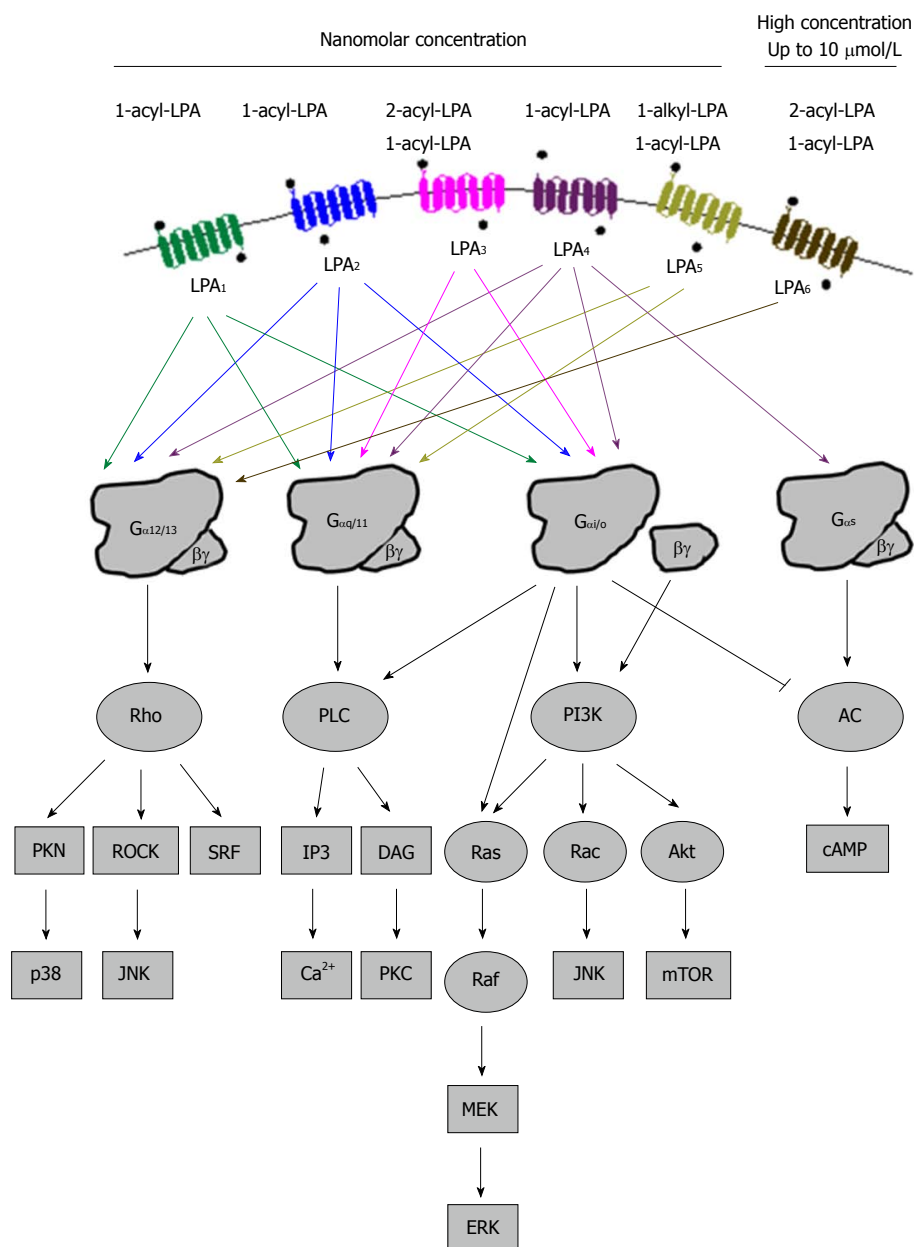
LPA acts as a potent mitogen, which was previously known as "ventricular zone gene-1 (vzg-1)" due its high level in the embryonic neuroproliferative layer of

the cerebral cortex<sup>[11,12]</sup>. The cloning and functional identification of LPA $_1$  led to determination of other receptor genes based upon sequence homology<sup>[81-83]</sup>. This is particularly true for the "endothelial differentiation gene" (EDG) members<sup>[84]</sup> that include LPA and sphingosine 1-phosphate receptors. Then, two other LPA receptors, LPA $_2$  and LPA $_3$  (also known as EDG4, and EDG7), were subsequently discovered based on shared homology with LPA $_1$ (EDG2)<sup>[85]</sup>. Later on, LPA $_4$  (P2RY9, GPR23)<sup>[86]</sup>, LPA $_5$  (GPR92)<sup>[87]</sup> and LPA $_6$  (P2RY5, GPR87)<sup>[88]</sup> were identified. They share 35% amino acid homology to the purinergic (P2Y) family of GPCRs, as compared to less than 20% homology to LPA $_1$ , suggesting that LPA $_{4-6}$  are more closely related to the P2Y receptors<sup>[52]</sup>. Here, LPA $_1$ -LPA $_6$  are for proteins, and their gene symbols are *LPAR1-LPAR6* for human and *Lpar1-Lpar6* for non-human<sup>[89]</sup>.

All LPA receptors signal through at least one of the four heterotrimeric G $\alpha$  proteins (G $\alpha_{12/13}$ , G $\alpha_{q/11}$ , G $\alpha_{i/o}$ , and G $\alpha_s$ )<sup>[12,90]</sup>, resulting in downstream signals that produce diverse physiological and pathophysiological effects (Figure 3). G $\alpha_{12/13}$ -mediated LPA signaling regulates cytoskeletal remodeling, cell migration and invasion through activation of Rho pathway proteins<sup>[91]</sup>. Rho signals to c-jun N-terminal kinase (JNK) and p38 through Rho-associated kinase (ROCK) and protein kinase N. The LPA-coupled G $\alpha_{q/11}$  protein primarily regulates Ca $^{2+}$  homeostasis through phospholipase C (PLC), which generates the second messengers IP $_3$  and diacylglycerol (DAG)<sup>[92-94]</sup>. G $\beta\gamma$  and G $\alpha_{i/o}$  subunits mediate the activation of phosphatidylinositol 3-kinase (PI3K) which results in the stimulation of the Akt pathway and increase of protein translation after the activation of the mammalian target of rapamycin (mTOR) signaling pathway. Activation of PI3K by G $\beta\gamma$  subunits also stimulates the activity of Rac, leading to cell migration and JNK regulation of pro-inflammatory gene expression, and Ras activity, leading to the stimulation of Raf- mitogen-activated protein kinase (MEK)-extracellular signal-regulated kinase (ERK) pathway to promote the expression of genes involved in proliferation and invasion. G $\alpha_{i/o}$ , besides PI3K, also stimulates the Ras-Raf-MEK-ERK pathway promoting cell survival and other functions<sup>[95,96]</sup>. G $\alpha_s$  can activate adenylyl cyclase and increase cAMP concentration upon LPA stimulation<sup>[97]</sup>. However, the same enzyme is also inhibited by G $\alpha_{i/o}$ , showing the complexity of signaling pathways after the activation of LPA receptors<sup>[98]</sup>.

All six LPA receptors can be stimulated by 1-acyl-LPAs, which show different potencies. LPA $_3$  and LPA $_6$  prefer unsaturated 2-acyl-LPA, while LPA $_5$  likes ether-linked 1-alkyl-LPA species<sup>[99,100]</sup>. In addition, lysophosphatidylserine, lysophosphatidylinositol, and lysophosphatidylethanolamine, have been thought to activate these receptors as well<sup>[101]</sup>. Different LPA molecules may have preference to different subtypes of LPA receptors<sup>[102]</sup>. Table 1 summarizes PLA receptors expression profiles and their known physiological functions in humans and mice.





**Figure 3 Summary of lysophosphatidic acid activated intracellular signaling pathways via the six cognate lysophosphatidic acid receptors.** PLC: Phospholipase C; PI3K: Phosphatidylinositol 3-kinase; AC: Adenyl cyclase; PKN: Polynucleotide 5'-hydroxyl-kinase; ROCK: Rho-associated kinase; JNK: c-jun N-terminal kinase; SRF: Serum response factor; IP3: Inositol 1,4,5-triphosphate; DAG: Diacylglycerol; PKC: Protein kinase C; MEK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinase; Akt: Protein kinase B; Mtor: Mammalian target of rapamycin.

### LPA<sub>1</sub>

LPA<sub>1</sub> is the first LPA receptor identified based on studies of LPA in the brain<sup>[11]</sup>. LPA<sub>1</sub> couples to three G $\alpha$  proteins - G $\alpha$ <sub>12/13</sub>, G $\alpha$ <sub>q/11</sub>, and G $\alpha$ <sub>i/o</sub>, which can result in the activation of downstream pathways including Akt, Rho, Ras, and PLC (Figure 3). These pathways mediate many cellular responses initiated by LPA<sub>1</sub> such as neurodevelopment regulation, cell proliferation, differentiation, apoptosis and survival, cell-cell contact through a variety of mechanisms<sup>[68,84,103-106]</sup>. *Lpar1*<sup>-/-</sup> mice exhibit about 50% perinatal lethality, which was attributed to the defective development of olfaction. The survived ones had reduction of body size, craniofacial dysmorphism, and loss of Schwann cells<sup>[107]</sup>. Dysregulation at glutamatergic

synapses was observed in *Lpar1*<sup>-/-</sup> mice<sup>[108]</sup>. When the original *Lpar1*<sup>-/-</sup> mouse line was expanded, a spontaneous variant named "Málaga LPA1" arose. They showed more severe brain defects than the original *Lpar1*<sup>-/-</sup> line mice did<sup>[109]</sup>. The loss of LPA<sub>1</sub> in animals seems to modulate the development of several diseases including cancer, obesity, neuropathic pain, fibrosis and male infertility<sup>[110]</sup>.

### LPA<sub>2</sub>

The amino acid sequence of LPA<sub>2</sub> is about 50% identical to that of LPA<sub>1</sub>, and it associates with G $\alpha$ <sub>i/o</sub>, G $\alpha$ <sub>q/11</sub>, and G $\alpha$ <sub>12/13</sub>, the same as LPA<sub>1</sub><sup>[106]</sup> (Figure 3). These G proteins use Ras, PI3K/Rac, PLC/DAG and Rho to mediate their

**Table 1** Expression pattern of lysophosphatidic acid receptors and their known physiological functions in humans and mice

| Name             | Information   | Previous orphan names  | Major expression tissue (high to low level)  | Knockout effects in mouse   | Biological functions   | Ref.  |
|------------------|---|--|--|---|--|---|
| LPA <sub>1</sub> | Human chromosome locus 9q31.3; 41.1 kDa <sup>1</sup> ; 364 aa <sup>2</sup> ; Identity <sup>3</sup> 97.3%<br><br>Mouse chromosome locus 4, 32.2 cM; 41.1 kDa; 364 aa | <i>vzg-1, edg-2</i> , 39.4 kDa; 344 aa<br><i>mrec1.3, lpA1</i> | Brain, placenta, urinary bladder, uterus, testis, lung, small intestine, heart, stomach, kidney, spleen, thymus, and skeletal muscle.<br><br>Brain, heart, lungs, stomach, intestine, placenta, kidneys, spleen, uterus, testes. | Perinatal lethality, retarded growth, defective olfaction, reduced body size, craniofacial dysmorphism with blunted snouts, and increased apoptosis in sciatic nerve Schwann cells.   | Neurodevelopment regulation, cell proliferation, differentiation, apoptosis and survival, cell-cell contact through serum-response element activation, cell migration and cytoskeletal organization, Ca <sup>2+</sup> homeostasis, cAMP-regulated cellular processes and adenylyl cyclase inhibition | Yung <i>et al</i> <sup>[68]</sup> , 2014; Archbold <i>et al</i> <sup>[138]</sup> , 2014; Choi <i>et al</i> <sup>[139]</sup> , 2008; Anliker <i>et al</i> <sup>[103]</sup> , 2013; Sakai <i>et al</i> <sup>[104]</sup> , 2013; Wittpoth <i>et al</i> <sup>[88]</sup> , 1999; An <i>et al</i> <sup>[82]</sup> , 1998; Contos <i>et al</i> <sup>[107]</sup> , 2000; Contos <i>et al</i> <sup>[106]</sup> , 2000; Fukushima <i>et al</i> <sup>[84]</sup> , 2001.                                      |
| LPA <sub>2</sub> | Human chromosome 19p13.11; 39.1 kDa; 351 aa; Identity 83.5%<br><br>Mouse chromosome 8, 33.91 cM; 38.7 kDa; 348 aa   | <i>edg-4, lpA2</i>   | Leukocytes, testis, prostate, spleen, thymus and pancreas.<br><br>Kidney, testis, uterus, lung, stomach, spleen, thymus, postnatal brain, and heart.   | Normal  | Cell migration, viable and healthy, nervous system development and immune system regulation.   | Yung <i>et al</i> <sup>[68]</sup> , 2014; An <i>et al</i> <sup>[82]</sup> , 1998; Contos <i>et al</i> <sup>[106]</sup> , 2000b; Archbold <i>et al</i> <sup>[138]</sup> , 2014; Ohuchi <i>et al</i> <sup>[111]</sup> , 2008; Choi <i>et al</i> <sup>[110]</sup> , 2010; Valentine <i>et al</i> <sup>[140]</sup> , 2008; Xu <i>et al</i> <sup>[113]</sup> , 2004; Lai <i>et al</i> <sup>[112]</sup> , 2005; Contos <i>et al</i> <sup>[115]</sup> , 2002; Choi <i>et al</i> <sup>[139]</sup> , 2008. |
| LPA <sub>3</sub> | Human chromosomal locus 1p22.3; 40.1 kDa; 353 aa; Identity 91.2%<br><br>Mouse chromosome locus 3, 71.03 cM; 40.3 kDa; 354 aa  | <i>edg-7, lpA3</i>   | Heart, testis, prostate, pancreas, lung, ovary, and brain.<br><br>Lung, kidney, uterus, testis, small intestine, brain, heart, stomach, placenta, spleen, and thymus.  | Delayed embryo implantation, embryo crowding, and reduced litter size for female null mutants.  | Male and female reproductive physiology, inflammation, cell Ca <sup>2+</sup> homeostasis and cAMP regulation, vertebrate left-right patterning during embryogenesis.   | Yung <i>et al</i> <sup>[68]</sup> , 2014; Bandoh <i>et al</i> <sup>[83]</sup> , 1999; Im <i>et al</i> <sup>[116]</sup> , 2000; Contos <i>et al</i> <sup>[107]</sup> , 2000a; Zhao <i>et al</i> <sup>[117]</sup> , 2015; Ye <i>et al</i> <sup>[118]</sup> , 2010; Hama <i>et al</i> <sup>[119]</sup> , 2010; Lai <i>et al</i> <sup>[120]</sup> , 2012  |
| LPA <sub>4</sub> | Human chromosome Xq21.1; 41.9 kDa; 370 aa; Identity 98.4%<br><br>Mouse chromosome X region D; 41.9 kDa; 370 aa  | <i>P2Y9/GPR23</i>  | Ovaries, thymus, pancreas, brain, heart, small intestine, testis, prostate, colon, and spleen.<br><br>Heart, ovary, skin, thymus, and bone Marrow.   | Inhibition of its differentiation into osteoblasts in human mesenchymal stem cell line; For mouse: increased trabecular bone volume, number, and thickness; pericardial effusions, severe edema and hemorrhage, abnormally dilated blood and lymphatic vessels and lymph sacs, and impaired pericyte recruitment. | ROCK-dependent cell aggregation and N-cadherin-dependent cell adhesion, cAMP accumulation, differentiation of immortalized hippocampal progenitor cells, negatively cell motility regulation and osteogenesis.   | Yung <i>et al</i> <sup>[68]</sup> , 2014; Ohuchi <i>et al</i> <sup>[111]</sup> , 2008; Choi <i>et al</i> <sup>[110]</sup> , 2010; Liu <i>et al</i> <sup>[123]</sup> , 2010; Mansell <i>et al</i> <sup>[124]</sup> , 2010; Liu <i>et al</i> <sup>[125]</sup> , 2009; Sumida <i>et al</i> <sup>[126]</sup> , 2010; Yanagida <i>et al</i> <sup>[71]</sup> ; Lee <i>et al</i> <sup>[97]</sup> , 2007; Rhee <i>et al</i> <sup>[121]</sup> , 2006; Lee <i>et al</i> <sup>[122]</sup> , 2008             |
| LPA <sub>5</sub> | Human chromosome 12p13.31; 41.3 kDa; 372 aa; Identity 79.0%<br><br>Mouse chromosome 6, 59.21 cM; 41.4 kDa; 372 aa   | <i>GPR92</i>   | Spleen, heart, small intestine, placenta, colon, and liver.<br><br>Small intestine, lung, heart, stomach, colon, spleen, thymus, skin, liver, platelets, mast cells, gastrointestinal lymphocytes, and dorsal root ganglia.      | Reduced lung metastasis by melanoma cells.  | Neurite retraction, stress fiber formation, receptor internalization, water absorption, Ca <sup>2+</sup> mobilization and cAMP accumulation, LPA-induced release of chemokine ligand 4 in mast cells.  | Yung <i>et al</i> <sup>[68]</sup> , 2014; Lee <i>et al</i> <sup>[87]</sup> , 2006; Lee <i>et al</i> <sup>[141]</sup> , 2015; Amisten <i>et al</i> <sup>[142]</sup> , 2008; Lundquist <i>et al</i> <sup>[129]</sup> , 2011; Araki <i>et al</i> <sup>[130]</sup> , 2014; Lin <i>et al</i> <sup>[128]</sup> , 2010; Yanagida <i>et al</i> <sup>[143]</sup> , 2013  |

|                  |  |                  |  |               |  |  |
|------------------|--|------------------|--|---------------|--|--|
| LPA <sub>6</sub> | Human chromosome 13q14.2; 39.4 kDa; 344 aa; Identity 93.0%<br>Mouse chromosome 14, region D3; 39.4 kDa; 344 aa | P2Y <sub>5</sub> | Hair, skin.<br><br>Hair, immune cells. | Hypotrichosis | Hair development, increased intracellular Ca <sup>2+</sup> , reduced forskolin-stimulated cAMP accumulation, and ERK1/2 activation | Yanagida <i>et al</i> <sup>[144]</sup> , 2011; Yanagida <i>et al</i> <sup>[143]</sup> , 2013; Raza <i>et al</i> <sup>[135]</sup> , 2014; Dong <i>et al</i> <sup>[145]</sup> , 2014; Lee <i>et al</i> <sup>[141]</sup> , 2015; Lee <i>et al</i> <sup>[133]</sup> , 2009 |
|------------------|--|------------------|--|---------------|--|--|

<sup>1</sup>Molecular mass were obtained from UniProt<sup>[146]</sup>; <sup>2</sup>aa means amino acids; <sup>3</sup>Identities between human and mouse lysophospholipid receptors were calculated in UniProt<sup>[146]</sup>. *vzg-1*: Ventricular zone gene-1; *edg*: Endothelial differentiation gene.

down-stream signals, which may regulate cell survival and migration<sup>[107]</sup>. LPA<sub>2</sub> regulates cell survival and cell migration in the development of nervous system and functions of immune system<sup>[68,90,106,110,111]</sup>. The focal adhesion molecule thyroid receptor-interacting protein 6<sup>[112,113]</sup> and several PDZ-domain and zinc finger proteins<sup>[114]</sup> interact with LPA<sub>2</sub>. The PDZ-binding domain of LPA<sub>2</sub> regulates Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor 2 activity, and activates PLC-3 and Akt/ERK signaling pathways. These pathways stimulate cell migration, enhance survival, and alter gene expression, accounting for the functions attributed to LPA<sub>2</sub>. *Lpar2*<sup>-/-</sup> mice are viable and healthy, while those null for both *Lpar1* and *Lpar2* show features essentially consistent with those of *Lpar1*<sup>-/-</sup><sup>[115]</sup>. These data suggest functional redundancy of LPA<sub>2</sub> with LPA<sub>1</sub>.

### LPA<sub>3</sub>

*LPAR3/Lpar3* was cloned based upon homology to already identified LPA receptor genes using degenerated primers in a PCR-based cloning strategy<sup>[83,116]</sup>. LPA<sub>3</sub> couples with G<sub>αq/11</sub> and G<sub>αi/o</sub> to mediate adenylyl cyclase inhibition, PLC activation and Ca<sup>2+</sup> mobilization, and Ras activation<sup>[105]</sup> (Figure 3). LPA<sub>3</sub> prefers 2-acyl-LPAs containing unsaturated fatty acids<sup>[83]</sup>. It mediates the activation of a series of physiological processes such as male and female reproductive physiology, inflammation, cell Ca<sup>2+</sup> homeostasis and cAMP regulation<sup>[107,117-119]</sup>. LPA<sub>3</sub> appears to determine vertebrate left-right patterning during embryogenesis as downregulation of *Lpar3* or inhibition of LPA<sub>3</sub> activity disrupted patterning process in zebrafish<sup>[120]</sup>. *Lpar3*<sup>-/-</sup> mice are viable with no reported neural deficits, even though LPA<sub>3</sub> is found in the frontal cortex, hippocampus, and amygdala<sup>[83,116]</sup>. On the other hand, female *Lpar3*<sup>-/-</sup> mice have a delayed embryo implantation, and reduced litter size<sup>[117]</sup>.

### LPA<sub>4</sub>

The first so-called non-EDG LPA receptor was identified in 2003, and named as LPA<sub>4</sub>. It shares homology (approximately 20%) with LPA<sub>1-3</sub>, and it is more closer to the P2Y receptor family<sup>[86]</sup>. LPA<sub>4</sub> was identified by screening orphan receptors using calcium mobilization as a readout for ligand-induced signals. LPA<sub>4</sub> couples with G<sub>α12/13</sub>, G<sub>αq/11</sub>, G<sub>αi/o</sub> and G<sub>αs</sub><sup>[97]</sup>, and activates Rho/ROCK to induce neurite retraction and stress fiber formation<sup>[71,97]</sup> (Figure 3). It induces ROCK-dependent cell aggregation

and N-cadherin-dependent cell adhesion<sup>[71]</sup>. LPA<sub>4</sub> is only LPA receptor that activates G<sub>αs</sub> to induce cAMP level<sup>[97]</sup>. The activation of LPA<sub>4</sub> was thought to regulate the differentiation of immortalized hippocampal progenitor cells<sup>[121]</sup>. In addition, the activation of LPA<sub>4</sub> could inhibit LPA-induced cell migration, but LPA exposure increased lamellipodia formation and transwell movement of LPA<sub>4</sub> null cells, indicating an increased sensitivity<sup>[122]</sup>. It shows the ability of LPA<sub>4</sub> to negatively regulate cell motility and indicates that differential effects may be achieved by simultaneously expressing multiple LPA receptors. *LPAR4*-deficient human mesenchymal stem cells lost ability to differentiate into osteoblasts<sup>[123]</sup>. While adult *Lpar4*<sup>-/-</sup> mice appear grossly normal<sup>[122]</sup>, they exhibit increased trabecular bone volume, number, and thickness<sup>[124,125]</sup>. LPA<sub>4</sub> pathway seems to inhibit osteogenesis. *Lpar4*<sup>-/-</sup> mice had reduction of prenatal survival rate during embryo development, which is accompanied by changes such as pericardial effusions, severe edema and hemorrhage<sup>[126]</sup>.

### LPA<sub>5</sub>

LPA<sub>5</sub>, the fifth LPA receptor, was identified in 2006<sup>[87,127]</sup>. It shares about 35% homology with *LPAR4*, and 22% homology with *LPAR1-3*<sup>[87]</sup>. LPA<sub>5</sub> couples with G<sub>α12/13</sub> and G<sub>αq/11</sub>, which mediate neurite retraction, stress fiber formation, and receptor internalization in LPA<sub>5</sub>-expressing cell lines<sup>[87]</sup> (Figure 3). It also activates G<sub>αq/11</sub> to increase intracellular calcium mobilization, and cAMP accumulation via a non-G<sub>αs</sub> mechanism, suggesting the involvement of other G-proteins<sup>[87,127]</sup>. LPA<sub>5</sub> signaling may also affect intestinal water absorption<sup>[128]</sup>. This is achieved through the LPA-induced recruitment of Na<sup>+</sup>/H<sup>+</sup> exchanger 3 to the microvilli mediated by the interaction between LPA<sub>5</sub> and Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor 2. Additionally, LPA<sub>5</sub> is the main LPA receptor responsible for LPA-induced release of chemokine ligand 4 in mast cells<sup>[129]</sup>. Interestingly, LPA<sub>5</sub> in B16 melanoma cells, prefers alkyl-LPA (18:1) to acyl-LPA (18:1)<sup>[99]</sup>. *Lpar5*<sup>-/-</sup> null mice exhibit reduced lung metastasis by melanoma cells compared with wild type ones<sup>[130]</sup>.

### LPA<sub>6</sub>

The most recently identified LPA receptor is LPA<sub>6</sub>. It was first isolated from a chicken T cell library and named receptor 6H1 in 1993<sup>[131]</sup>, and then, renamed to P2Y<sub>5</sub> because of sequence homology with P2Y receptors in

1996<sup>[132]</sup>. LPA<sub>6</sub> couples with G $\alpha$ -protein G $\alpha_{12/13}$  (Figure 3). Its activation by LPA causes cAMP accumulation, changes in cell morphology, and guanosine 5'-3-O-(thio) triphosphate binding<sup>[71]</sup>. When LPA<sub>6</sub> was expressed together with a G $\alpha$  protein, LPA stimulation increased intracellular Ca<sup>2+</sup> level, and decreased forskolin-induced cAMP level and ERK activation in intestinal cells<sup>[133]</sup>. LPA<sub>6</sub> has been thought to be involved in familial hair loss<sup>[134,135]</sup>. Mutations of lipase member H and LPA<sub>6</sub> in patients with hypotrichosis are respectively associated with a decrease in LPA production and abnormal LPA<sub>6</sub> activation in cells<sup>[134,136,137]</sup>. These findings demonstrate the roles of LPA<sub>6</sub> and LPA signaling may be therapeutic targets for the treatment or prevention of human hair loss<sup>[138-146]</sup>.

## LPA RECEPTOR SIGNALING IN OBESITY AND INSULIN RESISTANCE

Recently, obesity has become major public health concern, particularly in the United States. According to 2015 Center of Disease Control and Prevention estimates, more than one-third of adults (34.9% or 78.6 million) and 17% of youth in the United States were obese in 2011-2014<sup>[147]</sup>. Obesity is associated with the development of chronic metabolic diseases including diabetes, heart disease, stroke, and some types of cancer. The long-term effects of being overweight correlate with premature death, cardiovascular disease, metabolic morbidities, and asthma, among other problems<sup>[148]</sup>. Both environmental factors and genetic factors contribute to the obesity development. Many factors modulate the propensity to accumulate fat in cells, including an increased ratio of adipocyte precursor cells to differentiated adipocytes<sup>[149]</sup>.

### LPA receptor signaling regulates adipogenesis

Obesity is associated with adipocyte hypertrophy and hyperplasia. Hypertrophy results in excessive TAG accumulation in adipocytes. Hyperplasia results in recruitment of new adipocytes *via* proliferation and differentiation. LPA was found to induce proliferation of 3T3F442A preadipocytes, indicating the role of LPA signaling in fat storage<sup>[150]</sup>. LPA stimulation increases the growth of 3T3F442A cells *via* LPA<sub>1</sub>, which activates the Ras-Raf-MEK-ERK pathway, and of the focal adhesion kinase<sup>[20,151]</sup>.

It has been reported that *Lpar1*<sup>-/-</sup> mice exhibited greater adiposity than the control mice without alteration of feeding behavior, despite of lowered body weight<sup>[107]</sup>. Interestingly, *Lpar1*<sup>-/-</sup> mice were resistant to diet-induced obesity that may result at least in part from alterations in leptin production<sup>[64]</sup>. Mature adipocytes express more ATX than preadipocytes. When secreted from adipose tissue, ATX may promote preadipocyte proliferation. Its expression was up-regulated during adipocyte differentiation, and in *db/db* mice<sup>[44,45]</sup>.

The serum levels of LPC, the precursors of LPA,

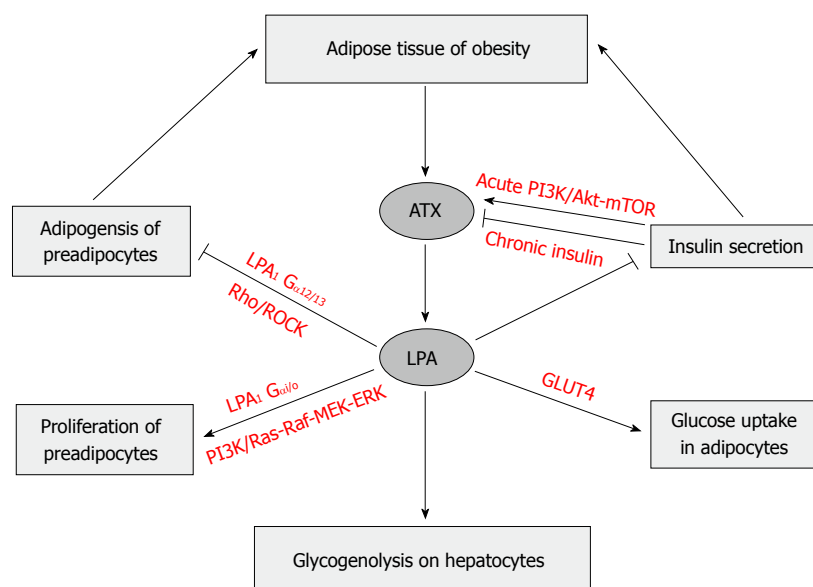
increases gradually in rabbits fed a high-cholesterol diet for 12 wk. The levels of individual LPAs formed after the incubation of serum for 24 h elevated with the increase of the length of time that rabbits were fed a high cholesterol diet<sup>[152]</sup>. These studies indicate that feeding of a high-fat diet can cause an increase in the circulating level of LPA. Preadipocytes mainly express LPA<sub>1</sub><sup>[153]</sup>, and the mRNA level of *Lpar1* expression in preadipocytes is higher than that in mature 3T3-L1 adipocytes<sup>[154]</sup>. However, in human adipose tissue, obesity does not influence on *LPAR1* expression<sup>[155]</sup>. This discrepancy of LPA<sub>1</sub> expression levels between human and mouse adipose tissues suggest that obesity promotes LPA synthesis rather than activation in adipose tissue.

The LPA-induced proliferation of preadipocytes<sup>[20,153,154]</sup> has been thought to be mediated through LPA<sub>1</sub> and the activation of the Ras-Raf-MEK-ERK pathway<sup>[154,156,157]</sup>. LPA inhibits differentiation of white and brown preadipocyte cell lines, which include porcine preadipocyte cell line; mouse preadipocyte cell line, 3T3-L1 and 3T3F442A; and human Simpson-Golabi-Behmel Syndrome preadipocyte cells<sup>[153,154,158,159]</sup>. This inhibition is mediated by LPA<sub>1</sub> *via* the Rho-ROCK pathway<sup>[160,161]</sup>. All these result in a down-regulation of PPAR $\gamma$ , and impaired responses of PPAR $\gamma$ -targeted genes to its ligands, which leads to reduced TAG accumulation, and expression levels of adipogenic genes<sup>[153,154]</sup>.

The activation of Rho-ROCK pathway delayed the activation of the Wnt-signaling pathway, which has been partially attributed to the inhibited PPAR $\gamma$  expression and adipogenesis. When mice with the adipocyte-specific knockout of ATX gene (FATX-KO) were fed a high-fat diet, they had more fat mass and larger adipocyte size, but not adipocyte number, than the control mice did in the absence of any change of food intake. The deletion of ATX in mice appeared to lead sensitivity to diet-induced obesity, which might be due to elevated expression levels of PPAR $\gamma$  and its down-stream adipogenic genes in subcutaneous white adipose tissue. Interestingly, those knockout mice had improved glucose tolerance and less systemic insulin resistance than the control mice fed the same diet<sup>[63,161]</sup>. LPA stimulation seems to have anti-adipogenic effect in white adipocytes<sup>[153]</sup> and in brown preadipocytes<sup>[159]</sup>. Aforementioned experiments seem to indicate that ATX-LPA receptor signaling pathway may inhibit the development of adipose tissue (Figure 4).

On the other hand, others reported that ATX promotes preadipocytes proliferation and differentiation into adipocytes, thereby promoting adipocyte hyperplasia and obesity. It was showed that deletion of ATX results in smaller body weight gain, smaller fat pad weights and adipocyte numbers, less insulin resistance and glucose tolerance in heterozygous *Enpp2*<sup>+/-</sup> mice and adipocyte-specific FATX-KO mice fed a high-fat diet than their littermates controls<sup>[162]</sup>. Moreover, the FATX-KO improved brown adipose tissue function,





**Figure 4 Autotaxin-lysophosphatidic acid signaling axis regulates adipose tissue development and glucose homeostasis in obesity.** In adipose tissue, especially in mature adipocytes, the elevated expression of ATX leads to production of LPA and then induced proliferation of preadipocytes *via* LPA<sub>1</sub> through Ras-Raf-MEK-ERK pathway. On the other hand, LPA inhibits differentiation of white and brown preadipocytes, which is mediated by LPA<sub>1</sub> *via* the Rho-ROCK pathway. Short-term insulin treatment increases ATX secretion in adipocytes *via* PI3K/Akt-mTOR pathway, whereas long-term insulin treatment reduces ATX activity. LPA produced by ATX in obesity has a tonic inhibitory effect on glucose homeostasis through inhibition of insulin secretion in isolated pancreas islets, increase of glucose transport in myocyte and adipocytes *via* GLUT4 translocation in a PI3K dependent manner, and elevation of glycogenolysis in hepatocytes. ROCK: Rho-associated kinase; PI3K: Phosphatidylinositol 3-kinase; MEK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinase; Akt: Protein kinase B; mTOR: Mammalian target of rapamycin; GLUT4: Glucose transporter type 4.

increased energy expenditure, and improved systemic metabolism. Transgenic mice expressing the human ATX/ENPP2 gene under the control of  $\alpha_1$  antitrypsin gene promoter became sensitive to diet-induced obesity due to reduced expression of brown adipose tissue-related genes in peripheral white adipose tissue and accumulated significantly more fat without any change of locomotor activities, thermogenic profiles, and systemic metabolism<sup>[159]</sup>. In mice, ATX is highly expressed in visceral white adipose tissue and brown adipose tissue and is downregulated in adipose tissue hypertrophy<sup>[162]</sup>. In human, ATX expression is higher in subcutaneous than in visceral fat, and the latter fat pad in obese subjects has higher ATX expression level than that in non-obese subjects, which is correlated with leptin expression<sup>[155]</sup>. The circulating ATX levels correlated negatively with body mass index, and mRNA levels of ATX were reduced in subcutaneous fat from obese subjects<sup>[162]</sup>. Moreover, ATX expression in adipose tissues may be negatively regulated by LPA through a feedback regulatory mechanism, which may involve inflammatory cytokines such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$ <sup>[163]</sup>.

#### ATX-LPA receptor signaling axis exerts a negative effect on glucose homeostasis in obesity

Earlier studies found that increases in adipocyte size correlated with insulin resistance, and increased risk of type 2 diabetes<sup>[164,165]</sup>. The expression of ATX is increased in the adipose tissue of obese and insulin-resistant subjects and mice<sup>[44,155,166]</sup>. It has been shown that LPA also regulates glucose metabolism<sup>[167,168]</sup>.

LPA was found to enhance glucose uptake in a dose-dependent manner in both GLUT4myc L6 myotubes and 3T3-L1 adipocytes, a process that was attributed to the increase of GLUT4 translocation in a PI3K dependent manner. Moreover, the effect of LPA on glucose uptake was completely inhibited by pretreating cells with LPA<sub>1/3</sub> receptor antagonist Ki16425 and Gi inhibitor pertussis toxin<sup>[169]</sup>. LPA significantly lowered blood glucose levels in normal mice and streptozotocin-induced diabetic mice, suggesting the promotion of glucose usage, but not stimulation of insulin secretion<sup>[169]</sup>.

The elevation of ATX expression in adipocytes of *db/db* mice occurred simultaneously with the development of hyperglycemia, and only 3 wk after the emergence of hyperinsulinemia in them<sup>[166]</sup>. ATX expression was up-regulated by treatment with TNF $\alpha$ , and down-regulated by rosiglitazone in 3T3F442A adipocytes<sup>[166]</sup>. The upregulation of ATX expression in adipocytes of *db/db* mice seems to be associated with the emergence of hyperglycemia rather than fat accumulation or hyperinsulinemia<sup>[166]</sup>.

The plasma levels of LPC, as the precursor of LPA, are reduced in obese and type 2 diabetic mice, suggesting that it may regulate blood glucose level. This reduction may contribute to the impairment of glucose homeostasis<sup>[170]</sup>. Interestingly, adipocyte specific ATX knockout mice fed with a high-fat diet showed greater adiposity and better tolerance to glucose challenge than control mice<sup>[63]</sup>, suggesting a negative effect of LPA on glucose homeostasis. Similarly, LPA production appears to impair glucose disposal probably through a reduction

of plasma insulin as pharmacological inhibition of LPAR<sub>1/3</sub> activation improves glucose homeostasis in obese and prediabetic mice<sup>[171]</sup>. Another possibility is that the progression of diabetes affects ATX expression in adipose tissue<sup>[162]</sup>. It has been shown that treatments with high concentrations of glucose and insulin led to ATX secretion in adipocytes. Short-term insulin treatment increased ATX activity, whereas long-term insulin treatment reduced the levels of ATX mRNA and protein, and its activity<sup>[172]</sup>.

In humans, ATX expression in adipose tissue significantly increased in diabetes patients in contrast with obese-only subjects<sup>[158,166]</sup>. Its expression in subcutaneous fat is higher than that in visceral fat. Nevertheless, ATX in visceral, but not subcutaneous, fat of obese subjects is higher than that in non-obese patients<sup>[155]</sup>. Interestingly, the circulating ATX levels in the blood were reduced in obese subjects<sup>[162]</sup>. The females have higher blood ATX level than males<sup>[173]</sup>.

The variations of ATX expression were correlated with some clinical parameters. In obese patients, visceral fat ATX was positively correlated with diastolic arterial blood pressure, plasma leptin level, and expression levels of inducible nitric oxide synthase and apelin receptor<sup>[155]</sup>. In older and obese humans, plasma ATX correlated with fasting glucose, fasting insulin, and glucose level 2 h after an oral glucose tolerance test, and body mass index<sup>[173]</sup>. LPA produced by ATX in obesity has a tonic inhibitory effect on glucose homeostasis through inhibition of insulin secretion in isolated pancreas islets, increase of glucose transport in myocyte and adipocytes, and elevation of glycogenolysis in hepatocytes<sup>[164]</sup>. LPA was reported to activate glycogenolysis in hepatocytes *in vitro*<sup>[174]</sup>, suggesting that LPA's effects on glucose homeostasis may be mediated by the liver. All these indicate that LPA production *via* ATX and its receptors activation may impact glucose homeostasis (Figure 4).

## POSSIBLE ROLE OF LPA SIGNALING IN LIVER FIBROSIS

The liver plays a critical role in the control of glucose and lipid homeostasis. The disturbance of this homeostasis may lead to development of metabolic diseases such as type 2 diabetes<sup>[175]</sup> and nonalcoholic fatty liver disease (NAFLD)<sup>[176]</sup>. Liver fibrosis is a process that leads to the alteration of the hepatic architecture marked by the accumulation of proteins such as collagen in extracellular matrix. This is generally associated with the development of liver diseases such as NAFLD and hepatitis. If left untreated, the further development of these diseases and liver fibrosis will lead to cirrhosis, and liver failure, which needs liver transplantation for the treatment. Factors causing damages of hepatocytes result in activation of hepatic stellate cells (HSCs) and production of pro-inflammatory and pro-fibrotic factors, which will stimulate formation of accumulation of proteins in extracellular matrix<sup>[177]</sup>.

The injuries caused by nutritional and environmental factors alter liver structures and functions, which may lead to the liver fibrosis<sup>[178]</sup>. The hepatic matrix is remodeled by the inflammatory responses after liver injury. Upon the stimulation, the generation of the liver matrix such as collagen, elastin, hyaluronan, proteoglycans and fibronectin is elevated, which is followed by remodeling processes. All these are associated with the activation of HSCs, and the change of local architecture and the reduction of liver functions. Excessive production and accumulation of extracellular matrix in the liver results in fibrosis, which can lead to liver cirrhosis<sup>[178]</sup>. In addition to HSCs, other cells responsible for the fibrosis include fibrocytes from hematopoietic stem cells, portal fibroblasts, bone marrow derived mesenchymal cells, epithelial-mesenchymal transition and endothelial to mesenchymal transition<sup>[178]</sup>.

The excessive accumulation of lipids and alterations of their metabolism have been used to explain the etiology of type 2 diabetes, which is associated with profound changes of hepatic gene expression<sup>[175,179]</sup>. This alteration of hepatic lipid metabolism may cause the development of fibrosis. For example, the elevation of lipid peroxidation in zone 3 hepatocytes has been suggested with the development of fibrosis<sup>[180]</sup>. On the other hand, changes of fatty acid compositions in plasma phospholipids have been observed in subjects with fibrosis<sup>[181,182]</sup>. All these show that the alterations of plasma phospholipids in patients with metabolic diseases may play a role in the development of fibrosis.

It has been shown that serum ATX activity and LPA level increase with the development of liver fibrosis in patients with chronic hepatitis C<sup>[74,183-185]</sup>, and with cholestasis and pruritus<sup>[186,187]</sup>. The association of elevated plasma ATX level with chronic liver disease (CLD) in patients suggests a shorter overall survival in a 10-year follow-up study<sup>[185]</sup>. Moreover, the increased expression level of hepatic ATX mRNA was found in the majority of publically available CLD and hepatocellular carcinoma (HCC) microarray data sets, suggesting an association of ATX with liver pathophysiology<sup>[185]</sup>. ATX and LPA levels increased in the plasma of patients with hepatitis C virus (HCV) infection, and positively associate with liver fibrosis stages<sup>[183,184,188,189]</sup>. HCV infection may stabilize the activity of hypoxia inducible factor in a PI3K dependent manner, which may increase ATX expression, and in turn induce liver fibrosis<sup>[190]</sup>.

It has been shown that serum ATX level correlated with fibrosis grade, and is useful as its marker in liver fibrosis<sup>[191]</sup>. The higher expression level of *LPA2* mRNA has been associated with the poorer differentiation of HCC cells, and a higher *LPA6* mRNA level is associated with microvascular invasion of HCC. The high expression levels of *LPA2* and *LPA6* mRNA in HCC predict a high potential for malignancy. The elevated levels of *LPA6* and *LPA6* mRNA in conjunction with plasma ATX predict higher rate of recurrence after surgical removal of the tumors<sup>[192]</sup>. In addition, the plasma level of ATX

has been considered as a potential pathogenic factor and/or biomarker for nonalcoholic fatty liver disease in nondiabetic and obese women<sup>[193]</sup>. Moreover, the plasma ATX levels correlated with prognosis of cirrhosis (Child-Pugh score), showing the link of ATX and the severity of cirrhosis in patients with CLD<sup>[194]</sup>.

In rats, plasma LPA level and serum ATX activity were increased in liver injury and were correlated with severity of the damage; the former in relation to the extent of fibrosis, and the latter in relation to the extent of hepatocyte damage<sup>[186,195]</sup>. In mice, different hepatotoxic stimuli linked with the development of different forms of CLD were shown to stimulate hepatocyte ATX expression, leading to increased LPA production, HSCs activation, and signals for fibrosis development<sup>[185]</sup>.

LPA was first shown to stimulate rat HSCs proliferation through MAP kinase activation in 1998<sup>[196]</sup>. Then, LPA was shown to enhance HSCs contractility through modulation of cellular morphology and attachment to extracellular matrices *via* Rho-kinase<sup>[197,198]</sup>. LPA also inhibits the apoptosis of those cells through Rho/Rho kinase activation<sup>[199]</sup>, suggesting its involvement in the pathogenesis of liver fibrosis. Moreover, LPA was shown to induce nuclear translocation of inducible nitric-oxide synthase in hepatocytes<sup>[200]</sup>. These findings demonstrate the possible involvement of LPA in the development of liver fibrosis.

## CONCLUSION

LPA is a highly bioactive lipid mediator with a number of cellular sources and exerts its actions through a family of receptors coupling with GPCRs in various cell types. Here, we have discussed recent advances in pathways for extracellular and intracellular production of LPA, the functions as well as structural and biochemical properties of ATX and LPA receptors. For the past 20-30 years, the cloning and identification of proteins mediating LPA production and signal transduction pathways open a new field for us to understand relevance of these proteins in physiology and disease development. The association of LPA production and signal pathways with chronic metabolic diseases has been gradually realized. We have highlighted the roles of LPA signaling pathways in the obesity, insulin resistance and liver fibrosis.

The realization of the importance of LPA-mediated functions leads to more open questions begging for answers. (1) The regulations of enzymes involved in LPA synthesis and degradation pathways remain to be further investigated. Whether intracellularly produced LPA can cross the plasma membrane into the extracellular compartment is currently unclear. Additional enzymes or pathways for the production of LPA are still worth exploring. For example, phosphatidylglycerol was shown to be converted to LPA under the catalytic action of GPAT as reported<sup>[201]</sup>. (2) For ATX, an important player for the extracellular LPA production, how its activity is regulated, and how the newly produced LPA is released remain to be

addressed. Future analysis will undoubtedly shed some light on these. (3) There are many factors contributing to the pathophysiology of obesity and metabolic diseases. Therefore, the precise role of LPA signaling pathways in these diseases remains to be investigated further. In addition, mechanisms by which the LPA and its receptor signaling pathways in the differentiation of both white and brown adipocytes remain to be clarified. This may help for the control of lipid metabolism. (4) LPA seems to have a negative effect on glucose homeostasis in obesity. This was observed only in obese patients, but not in non-obese subjects. So future human studies should focus on more healthy subjects and compare with those parameters of obese patients. (5) LPA appears to inhibit insulin secretion. Whether this inhibitory effect is due to a direct action of LPA on pancreas islets or a possible regulation of liver glycogen mobilization and/or muscle glucose oxidation remains to be clarified. And (6) It was shown that the plasma LPA level and serum ATX activity both were increased in association with liver fibrosis. The underlying mechanism remains to be determined.

Taken together, the LPA signaling pathways contain multiple points that potentially involve in the development of obesity, liver fibrosis and related pathologies. The development of novel pharmacological modulators targeting intervention points may open new research fields and provide potential medicinal therapies to reduce human suffering. The prospects are bright for expanding insights and contributions in LPA biology.

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## Ten years of sorafenib in hepatocellular carcinoma: Are there any predictive and/or prognostic markers?

Giorgia Marisi, Alessandro Cucchetti, Paola Ulivi, Matteo Canale, Giuseppe Cabibbo, Leonardo Solaini, Francesco G Foschi, Serena De Matteis, Giorgio Ercolani, Martina Valgiusti, Giovanni L Frassinetti, Mario Scartozzi, Andrea Casadei Gardini

Giorgia Marisi, Paola Ulivi, Matteo Canale, Serena De Matteis, Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola 47014, Italy

Alessandro Cucchetti, Leonardo Solaini, Giorgio Ercolani, Department of Medical and Surgical Sciences, Alma Mater Studiorum, University of Bologna, Bologna 40126, Italy

Alessandro Cucchetti, Leonardo Solaini, Giorgio Ercolani, General and Oncologic Surgery, Morgagni-Pierantoni Hospital, Forlì 47121, Italy

Giuseppe Cabibbo, Section of Gastroenterology, D.I.B.I.M.I.S., University of Palermo, Palermo 35628, Italy

Francesco G Foschi, Department of Internal Medicine, Degli Infermi Hospital, Faenza 48018, Italy

Martina Valgiusti, Giovanni L Frassinetti, Andrea Casadei Gardini, Department of Medical Oncology, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori IRCCS, Meldola 47014, Italy

Mario Scartozzi, Department of Medical Oncology, University of Cagliari, Cagliari 45698, Italy

ORCID number: Giorgia Marisi (0000-0003-4012-0042); Alessandro Cucchetti (0000-0001-5269-1964); Paola Ulivi (0000-0002-4334-9662); Matteo Canale (0000-0002-9768-7149); Giuseppe Cabibbo (0000-0002-0946-3859); Leonardo Solaini (0000-0002-5031-9285); Francesco G Foschi (0000-0002-8216-0918); Serena De Matteis (0000-0001-9219-4081); Giorgio Ercolani (0000-0003-4334-5167); Martina Valgiusti (0000-0002-9843-3538); Giovanni L Frassinetti (0000-0001-9376-0057); Mario Scartozzi (0000-0001-5977-5546); Andrea Casadei Gardini (0000-0001-6289-7202).

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**Correspondence to:** Alessandro Cucchetti, MD, PhD, Adjunct Professor, Surgeon, Department of Medical and Surgical Sciences, Alma Mater Studiorum, University of Bologna, Via Albertoni 15, Granarolo Dell'Emilia, Bologna 40126, Italy. [aleqko@libero.it](mailto:aleqko@libero.it)  
Telephone: +39-543-731111  
Fax: +39-543-739123

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### Abstract

Sorafenib has been considered the standard of care for patients with advanced unresectable hepatocellular carcinoma (HCC) since 2007 and numerous studies

have investigated the role of markers involved in the angiogenesis process at both the expression and genetic level and clinical aspect. What results have ten years of research produced? Several clinical and biological markers are associated with prognosis. The most interesting clinical parameters are adverse events, Barcelona Clinic Liver Cancer stage, and macroscopic vascular invasion, while several single nucleotide polymorphisms and plasma angiopoietin-2 levels represent the most promising biological biomarkers. A recent pooled analysis of two phase III randomized trials showed that the neutrophil-to-lymphocyte ratio, etiology and extra-hepatic spread are predictive factors of response to sorafenib, but did not identify any predictive biological markers. After 10 years of research into sorafenib there are still no validated prognostic or predictive factors of response to the drug in HCC. The aim of the present review was to summarize 10 years of research into sorafenib, looking in particular at the potential of associated clinical and biological markers to predict its efficacy in patients with advanced HCC.

**Key words:** Biomarker; Angiopoietin; Neutrophil-to-lymphocyte ratio; Polymorphisms; Sorafenib; MicroRNA; Adverse events; Hepatocellular carcinoma; Vascular endothelial growth factor

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**Core tip:** Sorafenib has been considered the standard of care for patients with advanced unresectable hepatocellular carcinoma, but after 10 years of research into sorafenib response or resistance, there are still no validated prognostic or predictive factors of response.

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## INTRODUCTION

Sorafenib, an oral multikinase inhibitor, has been considered the standard of care for patients with advanced unresectable hepatocellular carcinoma (HCC) since 2007<sup>[1]</sup>. It works by inhibiting the activity of several tyrosine kinases involved in tumor angiogenesis and progression, including vascular endothelial growth factor receptor (VEGFR-2/3), platelet-derived growth factor receptor (PDGF-R), Flt3 and c-Kit, and also targets Raf kinases involved in the MAPK/ERK pathway<sup>[2]</sup> (Figure 1). The molecular mechanisms by

which sorafenib exerts its activity have still not been fully elucidated, and both Raf/MEK/ERK-dependent and -independent mechanisms have been observed<sup>[3]</sup>.

Sorafenib is expensive and associated with adverse events (AEs). Furthermore, a proportion of treated patients show no response to the drug. It would thus be useful to have predictive markers capable of identifying those who are more likely to benefit from therapy. The availability of more accurate predictive or prognostic factors would also help to spare potentially resistant patients from unnecessary toxicity.

Ten years have passed since sorafenib was first commercialized and about 2800 studies have been published on the kinase inhibitor. But how many associated prognostic and/or predictive markers have been identified? Numerous studies have focused on the role of markers involved in the angiogenesis process at both the expression and genetic levels. The largest biomarker study conducted to date is the SHARP trial<sup>[4]</sup>, which included an adequate number of participants and a placebo-controlled group. Smaller single-arm studies exploring predictive or prognostic markers for sorafenib have also been conducted, but the results of these have yet to be validated.

The aim of the present review was to summarize 10 years of research into sorafenib, looking in particular at the potential of associated clinical and biological markers to predict its efficacy in patients with advanced HCC (Tables 1 and 2).

## CLINICAL PARAMETERS

### Alpha-fetoprotein

Alpha-fetoprotein (AFP) is secreted by about 50% of all HCCs and is the main serological marker used for the diagnosis of the tumor<sup>[5]</sup>. The SHARP trial<sup>[4]</sup> showed that high baseline AFP plasma levels (> 200 ng/mL) had a negative impact on overall survival (OS), a finding recently confirmed in a pooled analysis of the SHARP trial and the Asia Pacific trial by Bruix *et al.*<sup>[6]</sup>. High baseline serum AFP levels (≥ 400 ng/mL) also appear to be associated with shorter time-to-progression (TTP). Notably, in an analysis of six prospective phase II trials evaluating systemic therapies for patients with advanced HCC, no association was observed between baseline AFP levels and prognosis<sup>[7]</sup>.

Several studies<sup>[8-10]</sup> have highlighted a consistent correlation between an early decrease of > 20% in AFP levels following sorafenib and objective response and better outcome in advanced HCC patients. Shao *et al.*<sup>[8]</sup> evaluated for the first time this aspect and they observed that patients with early AFP response had an improved progression-free survival (PFS) (7.5 mo vs 1.9 mo) and OS (15.3 mo vs 4.1 mo). This data was confirmed by Personeni *et al.*<sup>[10]</sup> a few years later. They reported that early responders had a significantly better median OS and TTP than non-responders (13.8 mo vs 8.2 mo, *P* = 0.022 and 7.9 mo vs 2.4 mo, *P* = 0.004;



Table 1 Predictive and/or prognostic value of clinical markers in hepatocellular carcinoma patients

| Clinical markers                 | Predictive value | Prognostic value | Ref.       |
|----------------------------------|------------------|------------------|------------|
| Alpha-fetoprotein                | No               | Yes              | [6]        |
| Adverse events                   |                  |                  |            |
| Hand-foot skin reaction          | No               | Yes              | [13]       |
| Hypertension                     | No               | Uncertain        | [16,19,20] |
| Diarrhea                         | No               | Yes              | [21]       |
| Child-Pugh A vs B                | No               | Yes              | [27-29]    |
| Macroscopic vascular invasion    | No               | Yes              | [6]        |
| BCLC B vs C                      | No               | Yes              | [6,29,32]  |
| Starting dose and dose reduction | No               | Yes              | [29,32]    |
| Etiology HCV vs HBV              | Yes              | Yes              | [6]        |
| Chronic treatment with metformin | No               | Yes              | [35,36]    |
| Neutrophil-to-lymphocyte ratio   | Yes              | Yes              | [6,41,44]  |
| Extra hepatic spread             | Yes              | Yes              | [6]        |

HCC: Hepatocellular carcinoma; BCLC: Barcelona Clinic Liver Cancer; HCV: Hepatitis C virus; HBV: Hepatitis B virus.

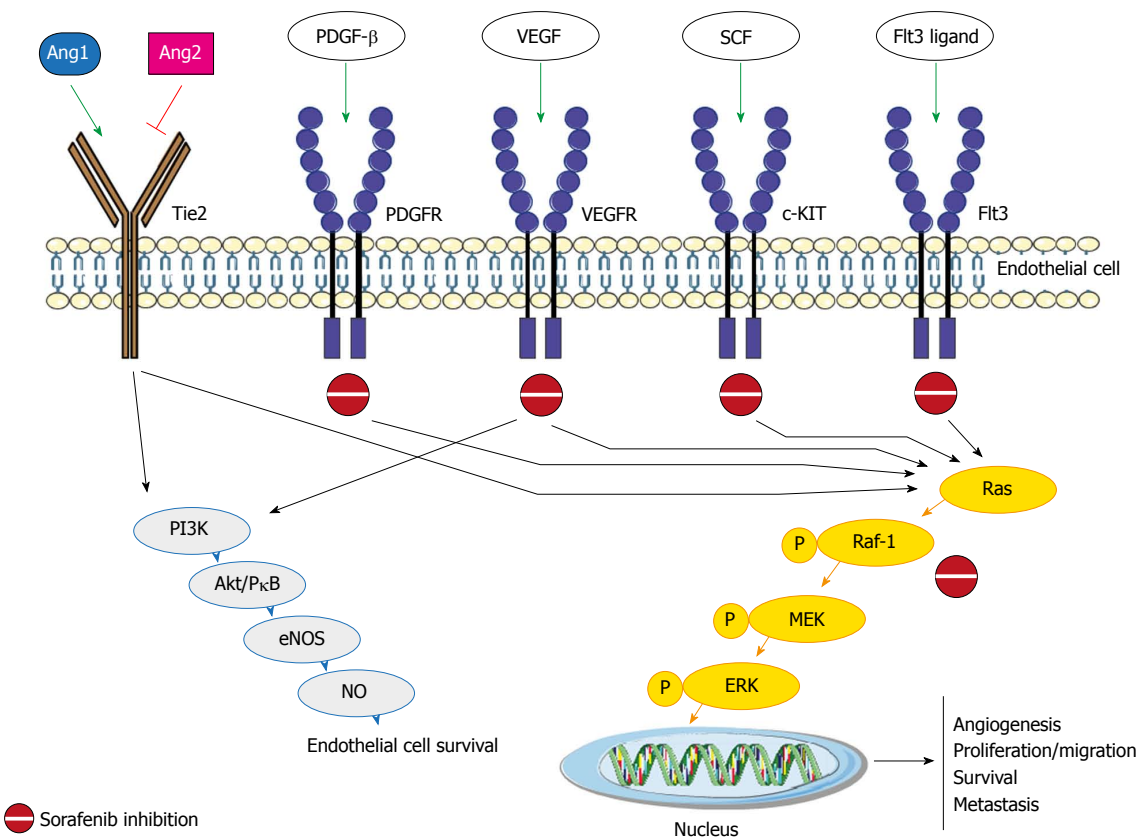


Figure 1 Sorafenib pathway and the main molecular factors. Ang: Angiopoietin; Tie2: Tyrosine-protein kinase receptor; PDGFR: Platelet-derived growth factor receptors; VEGFR: Vascular endothelial growth factor receptor; SCF: Stem cell factor; PI3K: Phosphatidylinositol 3-Kinase; Akt/PKB: Protein-kinase B; eNOS: Endothelial nitric oxide synthase; NO: Nitric oxide; P: Phospho-; MEK: Mitogen-activated protein kinase kinase; ERK: Extracellular signal-regulated kinase.

respectively). Conversely, Nakazawa *et al.*<sup>[11]</sup> did not observe such an association.

Adverse events

The main AEs of Sorafenib are hand-foot skin reaction (HFSR), hypertension and diarrhea. Several papers have highlighted a consistent correlation between AEs and survival in patients treated with Sorafenib.

Vincenzi *et al.*<sup>[12]</sup> evaluated for the first time the

correlation between HFSR and outcome. They showed, in a small series of patients treated with sorafenib, that patients with HFSR had a significantly higher disease control rate with respect to patients without HFSR. This data was confirmed in a prospective study of 147 patients by Reig *et al.*<sup>[13]</sup>. They reported different OS when patients were subdivided according to the presence or not of skin toxicity during the first 60 d of treatment (18.2 mo vs 10.1 mo, respectively)<sup>[13]</sup>. A

**Table 2** Predictive and/or prognostic value of biological markers in hepatocellular carcinoma patients

| Biological markers              | Predictive value | Prognostic value | Ref.    |
|---------------------------------|------------------|------------------|---------|
| Serum and plasma proteins       |                  |                  |         |
| VEGF-A                          | No               | Uncertain        | [4,57]  |
| Ang-2                           | No               | Yes              | [4]     |
| IGF-1                           | No               | No               | [55]    |
| Single nucleotide polymorphisms |                  |                  |         |
| VEGF-A rs2010963                | No               | Yes              | [65]    |
| VEGF-C rs4604006                | No               | Yes              | [65]    |
| eNOS (eNOS-786/eNOS VNTR)       | No               | Yes              | [66]    |
| Ang-2 rs55633437                | No               | Yes              | [67]    |
| HIF-1 $\alpha$ rs12434438       | No               | Yes              | [68]    |
| Amplifications                  |                  |                  |         |
| VEGF                            | No               | Uncertain        | [70]    |
| FGF3/FGF4                       | No               | Uncertain        | [71]    |
| miRNAs                          |                  |                  |         |
| miR-425-3p                      | No               | Yes              | [74]    |
| miR-224                         | No               | Yes              | [75]    |
| miR-181a-5p                     | No               | Yes              | [77]    |
| miR-339-5p                      | No               | Yes              | [77]    |
| miR-423-5p                      | No               | Yes              | [78]    |
| miR-10b-3p                      | No               | Yes              | [79]    |
| miR-221                         | No               | Uncertain        | [76]    |
| Tissue biomarker expression     |                  |                  |         |
| Phospho-ERK                     | Uncertain        | Uncertain        | [81,82] |
| PDGFR-b                         | No               | Yes              | [84]    |
| c-Met                           | No               | No               | [84]    |
| VEGFR                           | No               | No               | [84]    |
| p-c-Jun                         | No               | Yes              | [85]    |

Ang-2: Angiopoietin-2; IGF-1: Insulin-like growth factor-1; VEGF-A: Vascular endothelial growth factor A; HIF-1: Hypoxia-inducible factor 1; FGF: Fibroblast growth factor; miRNAs: MicroRNAs; eNOS: Endothelial nitric oxide synthase; PDGFR: Platelet-derived growth factor receptors; VEGFR: Vascular endothelial growth factor receptor; ERK: Extracellular signal-regulated kinase.

recently meta-analysis confirmed that HSFR was a good indicator of outcome for OS and TTP in HCC patients receiving sorafenib<sup>[14]</sup>

Hypertension (HTN) is frequently associated with the use of angiogenesis inhibitors<sup>[15]</sup>. Casadei Gardini *et al.*<sup>[16]</sup> showed that early HTN (15 d after the start of treatment) rather than later onset HTN vs patients without HTN was associated with better PFS (6.0 mo vs 2.5 mo;  $P < 0.001$ ) and OS (14.6 mo vs 3.9 mo;  $P = 0.003$ ). This finding has been confirmed in some studies<sup>[17,18]</sup> but not in others<sup>[19,20]</sup>.

Bettinger *et al.*<sup>[21]</sup> reported for the first time that diarrhea was an independent positive prognostic factor (HR = 0.41;  $P = 0.001$ ) in 112 patients with advanced HCC, a finding also confirmed by Koschny *et al.*<sup>[22]</sup>

Finally, other authors showed that the number of AEs was associated with predict survival in patients treated with sorafenib. In particular, Di Costanzo *et al.*<sup>[23]</sup> evaluated the potential of pretreatment clinical variables to predict survival. Three groups of patients were taken into account: patients without AEs (group 0), patients with one AE (group 1) and patients with two to three AEs (group 2). The study reported a strong correlation between this classification and disease progression at 3 mo (41.9%, 25.9% and 12.7% of patients in groups

0, 1 and 2, respectively;  $P = 0.014$ ). These data were subsequently confirmed in the validation cohort<sup>[24]</sup>. A recent meta-analysis by Abdel-Rahman *et al.*<sup>[25]</sup> revealed an association between specific side-effects (hypertension, HFSR and diarrhea) and patient outcome (HR = 0.38; 95%CI: 0.30-0.48;  $P < 0.00001$ ).

### Stage, liver functionality and etiology

**Child-Pugh A vs Child-Pugh B:** In the SHARP trial<sup>[4]</sup> and the Asia Pacific trial<sup>[26]</sup>, more than 95% of patients were classified as having Child-Pugh A cirrhosis, thus preventing the investigation of the potential benefits of sorafenib in Child-Pugh B patients.

Hollebecque *et al.*<sup>[27]</sup> reported for the first time the results from a prospective study on sorafenib efficacy in 120 advanced HCC patients, 20 of whom Child-Pugh B cirrhosis. OS was 11.1 mo, with a significantly longer median survival in Child-Pugh A patients than Child-Pugh B patients (13 mo vs 4.5 mo,  $P = 0.0008$ ). A few years later, Pressiani *et al.*<sup>[28]</sup> studied clinical outcome in a population of 300 consecutive patients; PFS in the Child-Pugh A group was 4.3 mo vs 2.1 mo in the Child-Pugh B arm (HR = 3.23; 95%CI: 2.38-4.39;  $P < 0.001$ ), TTP was 4.2 mo vs 3.8 mo and OS was 10.0 mo vs 3.8 mo, respectively ( $P < 0.001$ ).

The most important work on the use of sorafenib in Child-Pugh subgroups was the GIDEON study published in 2016<sup>[29]</sup>. This study observed that median OS was significantly longer in patients with Child-Pugh A (13.6 mo) than in those with Child Pugh B (5.2 mo) or Child-Pugh C (2.6 mo).

**Macroscopic vascular invasion:** It is widely acknowledged that the presence of macroscopic vascular invasion leads to a poorer prognosis<sup>[26,30]</sup>. The meta-analysis by Peng *et al.*<sup>[31]</sup> confirmed its prognostic value and the pooled analysis by Bruix *et al.*<sup>[6]</sup> affirmed the importance of macroscopic vascular invasion as a predictor of survival but not of response to treatment.

**BCLC stage:** In the SHARP trial, patients with Barcelona Clinic Liver Cancer (BCLC) B had a median OS of 14.5 mo compared to 9.7 mo for those with BCLC C<sup>[4]</sup>. Later, SOFIA<sup>[32]</sup> and GIDEON study<sup>[29]</sup> confirmed this data. In the SOFIA trial<sup>[32]</sup> the OS was 8.4 mo in BCLC C vs 20.6 mo in BCLC B patients ( $P < 0.0001$ ), but the time to radiologic progression did not differ significantly between the 2 groups. In the GIDEON study<sup>[29]</sup>, median OS according to BCLC by Child-Pugh cross-classification followed a similar trend, *i.e.* patients with Child-Pugh A and BCLC stage B showed longer OS than those with Child-Pugh B and BCLC B (19.5 mo vs 10.0 mo); and patients with Child-Pugh A and BCLC stage C had longer OS than those with Child-Pugh B and BCLC stage C (11.2 mo vs 3.8 mo).

Recently, Bruix *et al.*<sup>[6]</sup>'s pooled analysis confirmed that BCLC C patients had a poorer prognosis than those with BCLC B HCC (HR = 1.59;  $P = 0.02$ ).

**Sorafenib starting dose and dose escalation/reduction:** The two most important studies that evaluated sorafenib starting dose and dose escalation/reduction are SOFIA<sup>[32]</sup> and GIDEON trial<sup>[29]</sup>.

In the SOFIA trial<sup>[32]</sup> sorafenib was down-dosed in 161 (54%) patients because of AEs (133 patients, 83%) and a reduction in liver function (28 patients, 17%). Median OS of the 77 patients receiving a half-dose of sorafenib for 70% of the treatment period was 21.6 mo (95%CI: 13.6-29.6) compared with 9.6 mo (95%CI: 6.9-12.3) for the remaining 219 patients who had a dose reduction for  $< 70\%$  of the treatment period or who maintained the full dosage.

A sub-analysis of the GIDEON study<sup>[29]</sup> evaluated the starting dose of sorafenib with respect to clinical outcome and toxicity. Patients starting on 400 mg/d were slightly older, had baseline characteristics indicative of greater disease progression and had a higher incidence of AEs than those with a starting dose of 800 mg/d (96% vs 88%). Treatment duration (18.0 wk vs 13.0 wk) and median OS (12.1 mo vs 9.4 mo) were longer in patients receiving 800 mg/d.

**Etiology:** In the subgroup analysis of the SHARP

study<sup>[4]</sup>, the HR for OS was 0.76 in HBV-positive patients (95%CI: 0.38-1.50,  $P =$  not significant) and 0.50 (95%CI: 0.32-0.77) in HCV-positive patients. Results were similar for TTP (HR = 1.03 and 0.43 for HBV-positive and HCV-positive patients, respectively). Similar data were obtained for HBV-positive HCC patients in the phase III randomized Asia Pacific trial, *i.e.* the HR for OS was 0.74 (95%CI: 0.51-1.06, not significant) with respect to patients with the other etiology, for which the HR was 0.57 (95%CI: 0.29-1.33)<sup>[26]</sup>. Bruix *et al.*<sup>[6]</sup>'s pooled analysis of the SHARP/Asia Pacific trial results showed that the absence of HCV was a potential prognostic factor for poorer OS (HR = 0.7,  $P = 0.02$ ). The same authors revealed that HBV-positive patients did not show a significant difference in treatment response with respect to their HBV-negative counterparts (HR = 0.78; 95%CI: 0.57-1.06) and OS (HR = 1.128,  $P = 0.4538$ ). We believe that the 2 etiologic groups respond differently to sorafenib and that further investigation is warranted in specific studies<sup>[33]</sup>.

**Metformin treatment:** Type 2 diabetes is a significant risk factor for the development of malignancies, including HCC<sup>[34]</sup>. Casadei Gardini *et al.*<sup>[35]</sup> published findings of reduced sorafenib efficacy in HCC patients treated chronically with or without metformin for type II diabetes mellitus (PFS 2.6 mo vs 5.0 mo, respectively; and OS 10.4 mo vs 15.1 mo, respectively). The same authors validated these data in a series of more than 250 cases<sup>[36]</sup>, also highlighting a possible role of sirtuin-3 in resistance to sorafenib<sup>[37]</sup>. Di Costanzo *et al.*<sup>[38]</sup> recently reported an increase in TTP and OS in diabetic with respect to non-diabetic HCC patients. However, no distinction was made between the different hypoglycemic therapies administered.

#### Immune inflammation indicators

Systemic inflammatory responses have been shown to reflect the promotion of angiogenesis, DNA damage and tumor invasion through an upregulation of cytokines<sup>[39]</sup>. Previous research revealed that lymphocytes play a crucial role in tumor defense by inducing cytotoxic cell death and inhibiting tumor cell proliferation and migration<sup>[40]</sup>. Consequently, several inflammation and immune-based prognostic scores, such as lymphocyte count, neutrophil-lymphocyte ratio (NLR), and systemic immune-inflammation index (SII), have been developed to predict survival and recurrence in cancers, including HCC. Casadei Gardini *et al.*<sup>[41]</sup> evaluated for the first time SII, NLR and platelet-lymphocyte ratio (PLR) in a small case series, observing that SII were independent prognostic factors for OS. Other studies showed that NLR was a significant independent risk factor for shorter survival<sup>[42,43]</sup>. NLR was also found to be an independent prognostic factor for both response and survival in Bruix *et al.*<sup>[6]</sup>'s pooled analysis and Lue *et al.*<sup>[44]</sup>'s retrospective

study on Spanish patients.

## IMAGING EXAMINATIONS

The response to sorafenib does not correlate with a change in lesion dimension, but it is more correlated with intralesional vascularization. For this reason, the RECIST criteria<sup>[45,46]</sup> usually used for tumor response evaluation is inappropriate to evaluate the response to sorafenib in patients with advanced HCC. The modified RECIST (mRECIST) appear more indicate for evaluation the response. They include vascularization and tumor arterial enhancement changes of the target lesion on computed tomographic (CT). Several studies have demonstrated the superiority of the mRECIST criteria with respect to the RECIST criteria in assessing the response to treatment with sorafenib<sup>[47]</sup>. Various functional imaging tools were proposed to evaluate the antiangiogenic effects, but none of these has entered normal clinical practice<sup>[48-52]</sup>. Finally, a recently study showed that texture features on pretreatment contrast material-enhanced CT images can help predict OS and TTP in these patients<sup>[53]</sup>.

## BIOLOGICAL PARAMETERS

### Serum and plasma proteins

Although plasma biomarkers are the best candidates for evaluating sorafenib efficacy, only the SHARP trial produced results with borderline significance<sup>[4]</sup>. Baseline angiopoietin-2 (Ang-2) and vascular endothelial growth factor-A (VEGF-A) plasma levels independently predicted survival in both the entire patient population and the placebo cohort. Conversely, none of the tested biomarkers significantly predicted response to sorafenib<sup>[4]</sup>. Insulin-like growth factor (IGF)-1 levels have been found to decrease in patients with cirrhosis of the liver or HCC<sup>[54]</sup>, and high pretreatment levels of IGF-1 predict better PFS and OS in advanced HCC patients receiving first-line antiangiogenic therapy<sup>[55]</sup>.

The role of serum cytokines as biomarkers for the prediction of sorafenib responses is interesting, in particular Kim *et al.*<sup>[56]</sup> developed a new prediction model for sorafenib response that combines relevant serum markers, tumor related factors, and cirrhosis-related factors in a scoring system.

**VEGF-A:** Llovet *et al.*<sup>[4]</sup> showed that, although baseline plasma VEGF-A concentrations did not exhibit a predictive value, low plasma VEGF-A was associated with improved prognosis (HR = 1.48, 95%CI: 1.08-2.03,  $P = 0.015$ ). However, other authors did not find any association between VEGF-A and prognosis in patients treated with sorafenib<sup>[57]</sup>. Tsuchiya *et al.*<sup>[58]</sup>'s analysis of plasma VEGF concentrations during sorafenib treatment revealed that a decrease in the protein 8 wk after the start of therapy predicted better overall survival

in advanced HCC patients (30.9 mo vs 14.4 mo;  $P = 0.038$ ).

**Ang-2:** In the presence of VEGF, Ang-2 destabilizes blood vessels, promotes vascular sprouting, and is associated with an invasive and metastatic cancer phenotype<sup>[59]</sup>. Llovet *et al.*<sup>[4]</sup> demonstrated that high baseline Ang-2 levels were correlated with more aggressive disease (HR = 1.58, 95%CI: 1.20-2.07,  $P = 0.001$ ). Moreover, levels of the protein increased during treatment in the placebo group, suggestive of poor outcome related to disease progression in this cohort, whereas they remained constant during treatment with sorafenib, reflecting the generally more favorable outcome of this group. Overall increased Ang-2 expression levels were associated with poorer outcome in both groups, suggesting that this marker could be useful in monitoring treatment response. In agreement with Llovet's study, Miyahara *et al.*<sup>[57]</sup> reported that high baseline Ang-2 serum levels were associated with poor outcome in advanced HCC patients receiving sorafenib (HR = 2.51, 95%CI: 1.01-6.57,  $P = 0.048$ ). Although these results indicate the potential prognostic value of Ang-2 in HCC, its role in predicting response to sorafenib remains to be verified.

**IGF-1:** Shao *et al.*<sup>[55]</sup> found that high-pretreatment serum levels of IGF-1 were associated with a better DCR and improved PFS and OS in patients undergoing antiangiogenic therapy. Although the study did not have a control arm, the substantial significant difference in DCR between patients with high and low levels of IGF-1 (71% vs 39%) denotes the potential usefulness of IGF-1 as a predictive biomarker of response to antiangiogenic therapy.

**Multiple-factor analyses:** By using baseline serum basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) levels as covariates together etiology (B-viral), platelet count, BCLC stage and protein induced by vitamin K absence-II, Kim *et al.*<sup>[56]</sup> reported that a total score of < 6 could be a relevant cutoff value for selecting patients who are most likely to benefit from sorafenib therapy. Moreover, Hayashi *et al.*<sup>[60]</sup> found that serum interleukin (IL)-5, IL-8, CXCL9, PDGF-BB, TGF- $\alpha$ , and VEGF-A were elevated in the long survivors group among HCC patients who received sorafenib, potentially reflecting the activation of stromal signaling in the tumor microenvironment.

### Genetic markers

Molecular and genomic analyses from tumor and non-tumor tissue have proven useful in evaluating prognosis and could open up new avenues for tailoring treatment<sup>[61]</sup>. Genetic alterations, such as single nucleotide polymorphisms (SNPs) in genes encoding for proteins involved in the angiogenic process,



have been studied as potential biomarkers for anti-angiogenic therapy. SNP evaluation would seem to be more advantageous than protein or gene expression analyses as it can be performed at any time during the course of the disease, is not substantially influenced by laboratory biases, and is relatively inexpensive. Some authors have focused on molecular profiling in formalin-fixed paraffin-embedded (FFPE) samples, comparing the mutation profiles of HCC biopsy samples and the response to sorafenib treatment<sup>[62]</sup>. Gene amplification, gene mutations and expression profiling of tumors have now become a research priority and are expected to lead to personalized treatment for HCC patients<sup>[63,64]</sup>.

**SNPs:** Specific SNPs in *VEGF* and *VEGFR* genes have been found to be correlated with PFS and OS in HCC patients treated with sorafenib. In multivariate analysis, *VEGF-A* rs2010963 and *VEGF-C* rs4604006 were found to be independent factors influencing outcome in terms of PFS (HR = 0.25, 95%CI: 0.19-1.02,  $P = 0.0376$  and HR = 0.22, 95%CI: 0.14-0.81,  $P = 0.004$ , respectively) and OS (HR = 0.28, 95%CI: 0.23-0.96,  $P = 0.02$  and HR = 0.25, 95%CI: 0.17-0.99,  $P = 0.04$ , respectively)<sup>[65]</sup>. In the Italian multicenter, retrospective ePHAS [endothelial nitric oxide synthase (*eNOS*) polymorphisms in HCC and sorafenib] study, *eNOS* polymorphisms were analyzed in relation to PFS and OS. In univariate analysis, training cohort patients homozygous for *eNOS* haplotype (HT1:T-4b at *eNOS*-786/*eNOS* VNTR) showed a lower median PFS (2.6 mo vs 5.8 mo, HR = 5.43, 95%CI: 2.46-11.98,  $P < 0.0001$ ) and OS (3.2 mo vs 14.6 mo, HR = 2.35, 95%CI: 1.12-4.91,  $P = 0.024$ ) than those with other haplotypes. These results were confirmed in a validation set and multivariate analysis further substantiated this haplotype as the only independent prognostic factor<sup>[66]</sup>. More recently, evidence emerged that patients homozygous for *ANGPT2* (*Ang2* gene) rs55633437 GG genotype showed significantly longer PFS ( $P < 0.001$ ) and OS ( $P < 0.001$ ) than those with the other genotypes (GT+TT)<sup>[67]</sup>.

In the ALICE-2 study, Faloppi *et al.*<sup>[68]</sup> investigated the role of hypoxia-inducible factor 1- $\alpha$  (*HIF-1 $\alpha$* ) SNPs, confirming the results of the ALICE-1 study<sup>[65]</sup>. In multivariate analysis, rs12434438 of *HIF-1 $\alpha$* , rs2010963 of *VEGF-A* and rs4604006 of *VEGF-C* were confirmed as independent factors and may help to identify patients who are more likely to respond to sorafenib<sup>[68]</sup>. The prospective INNOVATE study is ongoing to validate the role of *VEGF*, *eNOS*, *Ang-2* and *HIF-1 $\alpha$*  SNPs in relation to clinical outcome in advanced HCC patients treated with sorafenib (NCT02786342)<sup>[69]</sup>.

**Gene amplification, gene mutations and RNA expression:** A relation between *VEGF-A* gene amplification and response to sorafenib was observed in a study performed on a mouse model of HCC<sup>[70]</sup>.

The authors found that HCC patients with tumor *VEGF-A* amplification showed markedly better survival than those with non-amplified tumors, highlighting that *VEGFA* amplification is a potential biomarker of response to *VEGF-A*-blocking drugs in HCC<sup>[70]</sup>. Arao *et al.*<sup>[71]</sup> observed that FGF3/FGF4 amplification and multiple lung metastases were frequently observed in responders to sorafenib, although the sample size was relatively small.

Sakai *et al.*<sup>[62]</sup> used targeted DNA and RNA sequencing in FFPE specimens from fine-needle biopsy to identify candidate biomarkers of response to sorafenib in 46 HCC patients. A significant difference was observed in the number of oncogene mutations between progressing and non-progressing patients ( $P = 0.045$ ), suggesting that tumor mutational burden may be predictive of sorafenib effectiveness. Tumor gene expression of NRG1, TGF $\alpha$ , and PECAM1 would also seem to be a marker of treatment response and PFS.

### MicroRNAs

MicroRNAs (miRNAs) affect drug response directly or indirectly by regulating the expression of genes involved in drug transportation, metabolism, and downstream signaling pathways. The deregulation of various miRNAs has been reported in *in vitro*, *in vivo* and population studies<sup>[72,73]</sup>, confirming its correlation with response to sorafenib. Some authors have evaluated the predictive role of miRNA expression in HCC tissue<sup>[74,75]</sup>, while others have studied circulating miRNA levels prior to sorafenib treatment<sup>[76]</sup>. To date, the most interesting tissue miRNAs are miR-425-3p<sup>[74]</sup> and miR-224<sup>[75]</sup>. High levels of miR-425-3p have been associated with longer TTP and PFS (HR = 0.4, 95%CI: 0.2-0.7,  $P = 0.0008$  and HR = 0.5, 95%CI: 0.3-0.9,  $P = 0.007$ , respectively), and elevated miR-224 expression have been correlated with increased PFS and OS (HR = 0.28, 95%CI: 0.09-0.92,  $P = 0.029$  and HR = 0.024, 95%CI: 0.07-0.79,  $P = 0.012$ , respectively). Circulating miRNAs have also been studied in the serum of HCC patients to predict early response to sorafenib treatment, with miR-181a-5p and miR-339-5p associated with partial response and disease progression<sup>[77]</sup>, miR-423-5p with stable disease or partial response<sup>[78]</sup> and miR-10b-3p with shorter survival<sup>[79]</sup>.

Another potentially interesting circulating miRNA is miR-221, which was studied by Fornari *et al.*<sup>[76]</sup> in both animal models and in a patient population. Patients with radiologic disease progression after 2-mo treatment had higher pretreatment miR-221 levels than responders ( $P = 0.007$ ).

Larger confirmatory studies are needed before miRNAs can be considered valid biomarkers for clinical practice.

### Tissue biomarker expression

A number of studies have analyzed tissue biomarkers

that may be very specific to the disease of interest<sup>[80]</sup>. In a phase II study of sorafenib in advanced HCC, Abou-Alfa *et al.*<sup>[81]</sup> showed that patients whose tumors expressed higher baseline phospho-ERK levels had a longer TTP. However, other studies reported conflicting results<sup>[82,83]</sup>. With regard to the expression of angiogenic markers in tumor tissue, it has been observed that high platelet-derived growth factor receptor beta expression is correlated with poor OS but not with PFS in HCC patients receiving sorafenib. High expression of the proto-oncogene c-Met may predict the therapeutic effectiveness of sorafenib in HCC patients, but no differences in terms of outcome have been seen with respect to VEGFR-2 expression<sup>[84]</sup>. Hagiwara *et al.*<sup>[85]</sup> studied another interesting tissue biomarker, phospho-c-Jun, reporting a significantly higher expression ( $P < 0.001$ ) in non-responding compared to responding patients treated with sorafenib.

## CONCLUSION

After 10 years of research into sorafenib, there are still no validated prognostic or predictive markers of response to sorafenib in hepatocellular carcinoma. Furthermore, the main results obtained to date come from 2 important randomized trials and from different subanalyses and pooled analyses rather than from normal clinical practice. The fact of there being only one drug for the treatment of these patients has certainly done nothing to stimulate research into identifying and validating predictors of response and prognosis. However, given the recent publication of a positive phase III trial<sup>[86]</sup> and the ongoing NCT01658878 immunotherapy study, the race is now on to see who will be the first to identify a prognostic and predictive factor for sorafenib and/or new drugs in this setting. In conclusion, the use of metabolomic profiling and whole genome analysis to examine the association between patient outcome and response to sorafenib could become alternative approaches to the search for new biomarkers in HCC.

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## Basic Study

# Differential expression of mucin 1 and mucin 2 in colorectal cancer

Aldona Kasprzak, Elżbieta Siodła, Małgorzata Andrzejewska, Jacek Szmeja, Agnieszka Seraszek-Jaros, Szczepan Cofta, Witold Szaflarski

Aldona Kasprzak, Elżbieta Siodła, Małgorzata Andrzejewska, Witold Szaflarski, Department of Histology and Embryology, Poznan University of Medical Sciences, Poznan 60-781, Poland

Jacek Szmeja, Chair and Department of General Surgery, Endocrinological and Gastroenterological Oncology, Poznan University of Medical Sciences, Poznan 60-355, Poland

Agnieszka Seraszek-Jaros, Department of Bioinformatics and Computational Biology, Chair of Clinical Pathomorphology, Poznan University of Medical Sciences, Poznan 60-529, Poland

Szczepan Cofta, Department of Pulmonology, Allergology and Respiratory Oncology, Poznan University of Medical Science, Poznan 60-569, Poland

ORCID number: Aldona Kasprzak (0000-0002-1773-3038); Elżbieta Siodła (0000-0003-2614-219X); Małgorzata Andrzejewska (0000-0003-4980-1725); Jacek Szmeja (0000-0003-0289-3511); Agnieszka Seraszek-Jaros (0000-0003-4787-1033); Szczepan Cofta (0000-0002-8849-5487); Witold Szaflarski (0000-0001-5059-268X).

**Author contributions:** Kasprzak A contributed to conception, design of the study, analyzed data and coordinated the research; Siodła E and Andrzejewska M performed the majority of immunohistochemical and molecular investigations; Szmeja J, Cofta S and Szaflarski W interpreted the data, analyzed the results, and performed a critical revision of the manuscript; Szmeja J provided samples of cancer patients and clinical data; Seraszek-Jaros A performed biostatistics and analyzed the data; Kasprzak A and Szaflarski W drafted the manuscript.

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**Correspondence to:** Aldona Kasprzak, MD, PhD, Professor, Department of Histology and Embryology, Poznan University of Medical Sciences, Swieczicki Street 6, Poznan 60-781, Poland. [akaspra@ump.edu.pl](mailto:akaspra@ump.edu.pl)  
**Telephone:** +48-61-8546441  
**Fax:** +48-61-8546440

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## Abstract

### AIM

To determine tissue expression (mRNA, protein) of two types of mucins [mucin 1 (MUC1) and mucin 2 (MUC2)] in patients with colorectal cancer (CRC).

### METHODS

Expression of membrane-bound mucin (MUC1) and secretory mucin (MUC2) in CRC (mRNA, protein) were analyzed in tissue material including fragments of tumors

obtained from CRC patients ( $n = 34$ ), and fragments of normal colorectal tissue from the same patients (control). The analysis was conducted using real-time quantitative polymerase chain reaction (RT-qPCR) (transcripts), immunohistochemistry (IHC) (apomucins), and the modern approach for morphometric analysis of IHC reaction (HSV filter software). Results on tissue expression of both mucins (mRNA, protein) were compared to histological alterations in colorectal cancer samples and correlated with selected clinical data in the patients. The statistical analysis was conducted using Statistica PL v. 12.0 software.

## RESULTS

Significantly higher expression of the MUC1 mRNA in the CRC, compared with the control and the borderline correlation of mRNA expression with MUC1 protein levels in colorectal samples was observed. The expression of apomucins concerned cell membranes (MUC1) and cytoplasm (MUC2) and occurred both in control tissues and in most cancerous samples. There were no significant relationships between MUC1 (mRNA, protein) and the clinicopathological data of patients. MUC2 protein expression was significantly lower as compared to the control, while MUC2 mRNA expression was comparable in both groups. The MUC1/MUC2 ratio was significantly higher in CRC tissues than in the control. The higher expression of MUC2 was a feature of mucinous CRC subtypes, and characterized higher histological stage of tumors. Negative correlations have been obtained between MUC2 and the Ki-67 antigen, as well as between MUC2 and p53 protein expressions in CRC.

## CONCLUSION

A combination of tissue overexpression of MUC1, reduced MUC2 expression, and high ratio of MUC1/MUC2 is a factor of poor prognosis in CRC patients. MUC2 tissue expression allows to differentiate mucinous and nonmucinous CRC subtypes.

**Key words:** Mucins; Real-time quantitative polymerase chain reaction; Colorectal cancer; Immunohistochemistry; HSV filter program

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**Core tip:** Colorectal cancers (CRC) represent the second most widely manifested malignant tumor worldwide in women and third in men. The evident expression of two mucins [mucin 1 (MUC1) and mucin 2 (MUC2)] occurs in a normal and cancerous large intestine. Using RT-qPCR analysis and immunohistochemistry we confirmed higher expression of the MUC1 mRNA, lower MUC2 protein, and higher MUC1/MUC2 expression ratio in CRC samples as compared to the control. MUC2 protein expression correlates with increased cellular proliferation. A combination of tissue overexpression of MUC1, reduced MUC2 expression, and high ratio of MUC1/MUC2 may be a useful factor of poor prognosis in CRC patients.

Jaros A, Cofta S, Szaflarski W. Differential expression of mucin 1 and mucin 2 in colorectal cancer. *World J Gastroenterol* 2018; 24(36): 4164-4177 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i36/4164.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i36.4164>

## INTRODUCTION

Colorectal cancer (CRC) is diagnosed in more than 1.3 million people worldwide, annually, with the number steadily increasing. Currently globally, this cancer is the third most common cancer in men and second most common in women<sup>[1]</sup>. In Poland CRC is the second most common cancer in men and woman, with the third leading causes of cancer deaths in Greater Poland Region<sup>[2]</sup>. While genetic factors play a major role in etiopathogenesis of CRC, the basis of most the cases of that cancer is unclear. Considering mutation source, CRC is classified as sporadic (70%), hereditary (25%) and congenital (3%-5%)<sup>[1,3]</sup>. Among the main pathologic alterations in CRC are quantitative and qualitative changes in glycoproteins called mucins<sup>[4-6]</sup>. Qualitative alterations of mucins include carbohydrate groups, as well as apomucin molecules<sup>[4,7-9]</sup>. The majority of CRC are nonmucinous adenocarcinomas (approximately 80%). A mucinous adenocarcinoma is a histological subtype of CRC with poorer prognosis than aforementioned. Quantitative changes identified in non-mucinous adenocarcinomas concern a reduction in total mucus output. In contrast, mucinous carcinomas are hypersecretory for mucus<sup>[4]</sup>.

According to modern proteomics, the secreted mucin, mucin 2 (MUC2) is the main constituent of intestinal mucus, produced mainly by the goblet cells of the small and large intestine and playing a critical protective role<sup>[4,10-12]</sup>. Membrane-associated mucin 1 (MUC1) (episialin), in contrast, is widely expressed by normal glandular epithelial cells, with its high expression in malignant cells<sup>[4,6]</sup>. Structural changes of the MUC1, observed in the course of carcinogenesis, lead to the activation of signaling pathways such as: MAPK, PI3K/Akt, and Wnt<sup>[6,13]</sup>. In the blood serum of cancer patients, the MUC1-N subunits, CA 15.3 and CA 19.9 antigens can be detected, while the MUC1 itself was second among the top 75 Tumor-Associated antigens<sup>[6]</sup>.

In the carcinogenesis initiation and CRC progression, overexpression of MUC1 and the decline in MUC2 expression is most commonly described<sup>[14-20]</sup>. These observations are also confirmed by meta-analysis<sup>[21-23]</sup>. However, knowledge of the role of tissue mucins expression, at various stages of the colon carcinogenesis is incomplete. Poorly known is the prognostic role of mucins in the mucinous subtypes of CRC, which generally have a worse clinical course and a worse response to chemotherapy<sup>[24,25]</sup>. Sporadic mucinous CRC had a worse survival rate than its nonmucinous counterpart<sup>[26]</sup>, and mucinous differentiation results in a 2%-8% increased hazard of death, which persists after correction for



stage<sup>[27]</sup>. Unlike the nonmucinous CRC, the mucinous subtype is correlated with higher MUC2 and lower MUC1 expression<sup>[4,21,24,28]</sup>. Research into the role of mucins in pathogenesis and CRC clinical studies (especially in mucinous subtypes) are also current topics from a methodological point of view. The lack of standardized methods of quantitative evaluation of mucins expression (especially at tissue level) and/or frequent lack of control groups, are a great difficulty in comparative analysis<sup>[15,29,30]</sup>.

The goal of the present work was the verification of the hypothesis, that the examination of the tissue expression of selected mucins (mRNA, protein), using modern methods of quantitative assessment [real-time quantitative polymerase chain reaction (RT-qPCR), HSV filter software], could improve the diagnostic/prognostic usefulness of these markers of CRC. The specific aim of the study was to evaluate tissue expression (mRNA, protein) of two mucins (MUC1 and MUC2) in patients with colorectal carcinoma, and to assess the relationship between tissue expression of mucins and selected clinicopathological data.

## MATERIALS AND METHODS

### Patients and tissue samples

The examined CRC group included 34 patients (27 men, 7 women) from Greater Poland Region, 32 to 89 years of age from the Chair and Department of General Surgery, Endocrinological and Gastroenterological Oncology, Poznan University of Medical Sciences, who were diagnosed and subjected to surgery between 2010-2015. We arbitrarily selected patients with CRC only from the Greater Poland Region, not treated before (radio- or chemotherapy), without significant additional systemic diseases, from whom consent was obtained, the peri-operative tissue material met the requirements for scientific research and with available clinicopathological data.

Patients affected by diabetes, active chronic organ diseases (heart, kidney, liver), including autoimmune diseases and other cancers, have been excluded from the study. In three patients, hyperglycemia was observed in fasting, four patients were in hypertension treatment. There have been mild premalignant lesions (mainly adenomatous colon), that have been surgically removed as a preventive measure in the past, in 14/34 (41%) of patients.

The available clinical data for the study group, than was taken into account, included: descriptive histopathological diagnosis, histologic grade and stage on Dukes, Astler and Collier's modified Dukes' scales, and TNM system classification<sup>[31,32]</sup>, age, patient sex and basic laboratory studies (complete blood count, number of leukocytes and platelets, as well as glucose levels). Seven patients (21%) of the entire study group died during the analysis period. Duration of patient's survival reflected the time between the date of operation for

colorectal cancer and the establishing diagnosis (October 1, 2010), and October 1, 2015.

Locations of the colorectal tumors were divided into proximal (right) colon (caecum, ascending, transverse colon) and distal (left) colon (descending, sigmoid colon and rectum). Macroscopic types were divided into protruded type (height of tumor  $\geq 3$  mm) and flat type (height of tumor  $< 3$  mm).

Thirty-four paired specimens of colorectal tumor and non-tumor tissues were obtained during surgical treatment. For the CRC, colon mucosa and, depending on the depth of tumor invasion, submucosal layers approximately 15 cm from the tumor site, served as control tissues. In no case was tissue additional to that which would be removed normally during a particular surgical procedure.

The tissue samples were stored in RNA Stabilization Solution (RNAlater®, Applied Biosystems) at  $-80^{\circ}\text{C}$  until use. Additionally, formalin-fixed paraffin-embedded tumor specimens of 34 colorectal carcinomas and fragments of the confirmed control specimens were obtained from patients.

Informed consent was obtained from every subject, and the institutional review committee approved this study (No. 924/14).

### RT-qPCR

CRC tumoral fragments and control tissues from 23 patients were qualified for the experiments that used the RT-qPCR technique as previously described<sup>[33]</sup>.

One microliter of given cDNA or DNA was added to the reaction mixture, composed of 12.5  $\mu\text{L}$  2  $\times$  Maxima® SYBR Green/ROX qPCR Master Mix (Fermentas), 1  $\mu\text{L}$  specific primer pair (*f.c.* 0.3  $\mu\text{mol/L}$ ) and 10.5  $\mu\text{L}$  H<sub>2</sub>O. Primers for studies on expression of *MUC1* and *MUC2* mRNA expression are indicated in Table 1.  $\beta$ -actin, glyceraldehyde-3-phosphate dehydrogenase (*GADPH*), and hypoxanthine-guanine phosphoribosyltransferase 1 (*HPRT1*) served as the housekeeping genes (geometric mean) for the gene expression analysis. All the primers were purchased from the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw (Table 1).

The reactions were driven in twin.tec real-time PCR plates with PCR Film (Eppendorf) using Mastercycler ep-realplex<sup>2</sup> (Eppendorf). The PCR program was as followed: (1) Initial denaturation,  $95^{\circ}\text{C}$ , 10 min; (2) Denaturation,  $95^{\circ}\text{C}$ , 15 s; (3) Annealing,  $60^{\circ}\text{C}$ , 30 s; (4) Extension,  $72^{\circ}\text{C}$ , 30 s. The number of cycles was 40-50. Melting curves were made and 2% agarose gel electrophoresis was used to verify the amplification product specificity and size, respectively. All samples were amplified in duplicate or triplicate, and in case when results varied by more than 15%, the reactions were repeated.

Absolute quantitation method was used to quantify mRNA copy numbers of *MUC1* and *MUC2*. Absolute quantification determines the exact copy concentration

**Table 1** Primer sequences used for real-time quantitative polymerase chain reaction analysis

| Transcript             | Sequence (5'-3' direction)                       | ENST number<br><a href="http://www.ensembl.org">http://www.ensembl.org</a> | Product size |
|------------------------|--|--|--------------|
| <i>MUC1</i>            | TCCAATATTAAGTTCAGGCCAGGA<br>CACATCACTCACGCTGACGT | 00000185499.16   | 768 bp       |
| <i>MUC2</i>            | TGAAGACCTGCGGCTGTGT<br>CAGTCGAACCTCGAAGTGCTCC    | 00000198788.8  | 3108 bp      |
| $\beta$ - <i>actin</i> | TCTGGCACCACACCTTCTAC<br>GATAGCACAGCCTGGATAGC     | 00000298556  | 169 bp       |
| <i>GADPH</i>           | GAAGGTGAAGGTCGGAGTCA<br>GACAAGCTTCCCGTTCTCAG     | 00000229239  | 199 bp       |
| <i>HRPT1</i>           | CTGAGGATTTGGAAGGGTG<br>AATCCAGCAGGTCAGCAAC       | 00000298556  | 156 bp       |

of a target gene by relating the  $C_t$  value to a standard curve. Prior to absolute quantification, the  $C_t$  values were normalized by comparison to the average of  $C_t$ 's obtained for three housekeeping genes ( $\beta$ -*actin*, *GADPH*, and *HRPT1*).

Evaluation of alterations in expression of MUC1 and MUC2 mRNA, involved a comparison of mRNA copy numbers for those mucins per microgram of RNA, between the tumor and control samples from the same patient.

#### Immunocytochemistry

Tissue sections, 5  $\mu$ m thick, were deposited onto SuperFrost/Plus microscope slides. In order to qualify the material for the study, routine staining of the sections with hematoxylin and eosin (HE) was performed. Anti-human mouse monoclonal antibodies (mAbs) specific for human Ki-67 antigen (clone MIB-1) (Dako Denmark A/S, Glostrup, Denmark, ready to use), anti-p53 (clone DO-7) (Dako), as well as the anti-MUC1 (clone Ma552) and anti-MUC2 (clone Ccp58) (both from Novocastra™, both in 1:100 dilution) antibodies were used. The sections were incubated with these primary mAbs through the night, at 4 °C, and afterwards with dextran backbone, to which horseradish peroxidase (HRP) was attached, and with secondary biotinylated link anti-rabbit and anti-mouse IgG (Dako REAL™ EnVision™ Detection System peroxidase/DAB+, Rabbit/Mouse, Dako), with microwave-oven pre-treatment for antigen retrieval. Positive reaction manifested, in at least three sequential sections, as a dark brown or black precipitate in the cell nucleus (Ki-67, p53) and cell membrane/cytoplasm (MUC1 and MUC2). The preparations were counterstained using hematoxylin. Every test was accompanied by a negative control, in which specific antibodies were supplemented by a normal serum of a respective species in 0.05 mol/L Tris-HCl, pH approximately 7.6, supplemented with 0.1% bovine serum albumin (BSA) and 15 mmol/L sodium azide (internal negative control). All the steps of immunocytochemistry (IHC) technique were previously described<sup>[34]</sup>. Histological slides with IHC expression were examined under the optical Olympus BH-2 microscope, coupled to a digital camera. Color microscope images were recorded and archived using a 40  $\times$  objective (at

least 10 fields in every microscope slide with an IHC positive reaction), with the use of LUCIA Image 5.0 computer software.

#### Semiquantitative evaluation of Ki-67 antigen and p53 expression

Expression of Ki-67 antigen and p53 (only clearly labelled cell nuclei were considered), was calculated, taking mean proportion of immunopositive cells in 10 light microscope fields into account. Expression was evaluated using the modified semi-quantitative scale<sup>[35]</sup>, in which the score of 1 corresponded to up to 10% positive cells; the scores of 2, 3 and 4 corresponded to 11%-25%, 26%-50% and  $\geq$  51% positive cells, respectively.

#### Morphometric evaluation of MUC1 and MUC2 tissue expression

The images with positive IHC reaction, 2560  $\times$  1920 pixels in size, recorded in the LUCIA Image 5.0 software, were subjected to morphometric analysis, using the quantitative morphometric HSV Filter software, originally developed in the Department of Bioinformatics and Computational Biology, Poznan University of Medical Sciences, according to the following formula: (area of positive IHC reaction/area studied)  $\times$  100%.

In the Results section, values of average IHC expression of both mucins were presented, expressed in percentages, manifested by the IHC reactions per field of colorectal cancer/control sample area.

#### Statistical analysis

At the first stage of statistical analysis, consistency of all of the results with normal distribution of Gauss was verified using the Shapiro-Wilk test. Parameters of descriptive statistics (mean value, median value, SD, and minimum and maximum value) were calculated.

Data related to quantitative mucin expression (mRNA, protein), in CRC group, were compared with the data obtained for the control samples of the same patients (linked variables) with the Wilcoxon test. In cases of unlinked variables in two groups, the non-parametric Mann-Whitney's test was applied. The *t*-Student test was applied in case of consistency of the results with normal Gaussian distribution.

**Table 2** Clinicopathologic features of colorectal carcinoma patients *n* (%)

| Variable                        |                                | CRC ( <i>n</i> = 34) |
|---------------------------------|--------------------------------|----------------------|
| Age (yr)                        | < 50                           | 2 (6)                |
|                                 | ≥ 50                           | 32 (94)              |
| Sex                             | male                           | 27 (79)              |
|                                 | female                         | 7 (21)               |
| Tumor location                  | Right colon                    | 10 (29)              |
|                                 | Left colon                     | 21 (62)              |
|                                 | Rectum                         | 3 (9)                |
| Mucin content                   | Nonmucinous                    | 24 (71)              |
|                                 | Mucinous                       | 10 (29)              |
| Histologic grade (G)            | Carcinoma <i>in situ</i>       | 1 (3)                |
|                                 | Well differentiated (G1)       | 1 (3)                |
|                                 | Moderately differentiated (G2) | 23 (68)              |
|                                 | Poorly differentiated (G3)     | 9 (26)               |
| Gross morphology                | Protruded                      | 21 (62)              |
|                                 | Flat                           | 13 (38)              |
| Dukes/ Astler and Collier stage | Carcinoma <i>in situ</i>       | 1 (3)                |
|                                 | A/B1                           | 4 (12)               |
|                                 | B/B2, B3                       | 10 (29)              |
|                                 | C/C1, C2, C3                   | 14 (41)              |
|                                 | D                              | 5 (15)               |
| TNM classification system       | Carcinoma <i>in situ</i>       | 1 (3)                |
|                                 | I and II                       | 4 (12)               |
|                                 | III and IV                     | 29 (85)              |
| Status                          | Survival                       | 27 (79)              |
|                                 | Death                          | 7 (21)               |

CRC: Colorectal carcinoma.

The percentage shares of IHC positivity of both mucins were evaluated, using the difference test between two proportions.

Correlations between data rows were determined employing Spearman's rank correlation index. The Kaplan-Meier survival curves and Log-rank test were used to compare overall survival rates. The results were accepted to be significant at the level of *P* value less than 0.05. The statistical analysis was conducted using Statistica PL v. 12.0 software (StatSoft Inc., Tulsa, OK, United States). The statistical analysis of the study was performed by biomedical statistician (AS-J). The statistical method of the study was reviewed by a statistician (Kaczmarek E) from the Department of Bioinformatics and Computational Biology, Chair of Pathology, Poznan University of Medical Science.

## RESULTS

### Clinicopathological data in CRC patients

Patients over 50 years of age were predominant in the Study Group (94%). The cancer was primarily located in the distal part of the colon (left colon) (62%). In 3 patients, the tumor was localized in the rectum. In 21 patients (62%), protruded type of tumor was observed, while the flat type was seen in 13 patients (38%).

The majority of patients were diagnosed with tubular adenocarcinoma located in the colon or rectum, and nonmucinous subtype of CRC (71%) prevailed. Among these patients one had a mixed-type tumor with the neuroendocrine component, the other was diagnosed as adenocarcinoma *in situ*. Mucinous subtype of CRC was

diagnosed in 10/34 patients.

The histopathological study showed a majority of moderately differentiated adenocarcinoma of the colon or rectum [grade 2 (G2)] (68%) compared to other grades. On a Dukes scale and in its modified form (Astler and Collier scale), most tumors were assessed at Stage C/C1-C3. In five patients from the whole Study Group (15%), there were distant metastases present (all to the liver). The vast majority of patients (85%) was classified stage III and IV on the TNM classification system.

The clinicopathological characteristics of CRC patients were collected in Table 2.

### MUC1 and MUC2 expression analysis at mRNA level

The expression of the MUC1 and MUC2 transcripts was present in all control and cancerous tissue samples. Our study showed that the expression of the MUC1 mRNA in the CRC tissues ( $75095 \pm 72149$  copies/ $\mu$ g RNA) was significantly higher when compared with the control tissue ( $32413 \pm 44486$  copies/ $\mu$ g RNA) ( $P = 0.004$ ), and the expression of MUC2 mRNA was comparable in the study and control group ( $350227 \pm 529270$  vs  $219744 \pm 324252$  copies/ $\mu$ g RNA) ( $P = 0.274$ ).

The MUC1/MUC2 transcripts ratio in the test group, although higher ( $1.56 \pm 4.50$ ), did not differ significantly from the one obtained in the control tissue ( $0.28 \pm 0.40$ ) ( $P = 0.128$ ) (Table 3).

### MUC1 and MUC2 mRNA expression and pathological data

No significant differences could be disclosed in the amount of MUC1 and MUC2 transcripts on one hand and

**Table 3** Tissue expression of mRNA and proteins of both mucins, mucin 1/mucin 2 ratio in colorectal carcinoma and in unaltered colorectal tissue

|                 |         | Group   | Number | Mean   | Median | Min  | Max     | SD     | <sup>a</sup> P value |
|-----------------|---------|---------|--------|--------|--------|------|---------|--------|----------------------|
| MUC1            | mRNA    | CRC     | 23     | 75095  | 49309  | 5648 | 267473  | 72149  | 0.004                |
|                 |         | Control | 23     | 32413  | 20075  | 2    | 199681  | 44486  |                      |
|                 | Protein | CRC     | 34     | 2.57   | 1.64   | 0.33 | 9.80    | 2.24   | 0.627                |
|                 |         | Control | 32     | 2.16   | 1.72   | 0.00 | 6.54    | 1.64   |                      |
| MUC2            | mRNA    | CRC     | 23     | 350227 | 191457 | 2806 | 2399156 | 529270 | 0.274                |
|                 |         | Control | 23     | 219744 | 130691 | 1    | 1509936 | 324252 |                      |
|                 | Protein | CRC     | 34     | 4.94   | 2.15   | 0.20 | 32.30   | 7.24   | 0.035                |
|                 |         | Control | 32     | 7.40   | 5.15   | 0.73 | 31.60   | 6.77   |                      |
| MUC1/MUC2 ratio | mRNA    | CRC     | 23     | 1.56   | 0.25   | 0.06 | 21.30   | 4.50   | 0.128                |
|                 |         | Control | 23     | 0.28   | 0.18   | 0.04 | 2.00    | 0.40   |                      |
|                 | Protein | CRC     | 34     | 1.84   | 0.80   | 0.04 | 10.35   | 2.54   | 0.003                |
|                 |         | Control | 32     | 0.52   | 0.37   | 0.00 | 1.95    | 0.51   |                      |

Control: Unaltered colorectal tissue; <sup>a</sup>P: Comparing colorectal carcinoma and control. MUC1: Mucin 1; MUC2: Mucin 2; SD: Standard deviation; CRC: Colorectal carcinoma.

CRC subtype (mucinous vs nonmucinous), colon tumor size, anatomical location of the CRC (proximal vs distal section of the colon), histologic grade or stage in the Dukes, or Astler and Collier scale, on the other (data not shown).

The comparison of the mRNA expression of both mucins, depending on the parameters in the TNM classification system was possible only for patients with N0 and N1, with no significant differences observed in this case as well (data not shown).

#### **MUC1 and MUC2 expression at protein level**

Using immunohistochemistry, a positive MUC1 immunoexpression was detected in all CRC samples (100%) and in 29/32 control colorectal samples (91%), thus the detectability of the positive expression of both mucins was similar. The immunoexpression of MUC2 was present in all CRC samples and in all samples of the colorectal control.

#### **Tissue localization of MUC1 and MUC2 immunoexpression**

MUC1 tissue expression in CRC was pronounced and related mostly to cell membranes on the apical surface of the neoplastic cells lining the glandular structures and in the lumen of altered intestinal crypts (extracellular mucins fields) (Figure 1A and B). In the control tissue of large intestine, membranous expression of MUC1 prevailed and was observed mainly on the surface of normal intestinal crypts (Figure 1C).

In contrast, MUC2 expression was mainly related to the cytoplasm of neoplastic cells with differentiated expression of this mucin, from single immunopositive cells (Figure 1D) to intense reaction in the cytoplasm of numerous cancer cells and/or localized extracellularly (Figure 1E). In the normal intestinal mucosa (control), cytoplasmic expression of MUC2 prevailed and was observed in normal intestinal crypts (Figure 1F).

No preferred detection sites were observed for both mucins (MUC1 and MUC2) within the evaluated area of

the colorectal tumor (center, periphery).

#### **Quantitative analysis of MUC1 and MUC2 immunoexpression**

The mean expression of MUC1 in colorectal tumors ( $2.57\% \pm 2.24\%$  of IHC reaction) and in the normal large intestine ( $2.16\% \pm 1.64\%$ ) was comparable ( $P = 0.627$ ). In the case of MUC2, significantly lower expression of this glycoprotein in CRC ( $4.94\% \pm 7.24\%$ ) than in the control ( $7.40\% \pm 6.77\%$ ) has been shown ( $P = 0.035$ ) (Table 3).

The MUC1/MUC2 expression ratio was significantly higher in the CRC tissues ( $1.84 \pm 2.54$ ), than in the control ( $0.52 \pm 0.51$ ) ( $P = 0.003$ ) (Table 3).

#### **MUC1 and MUC2 immunoexpression and pathological data**

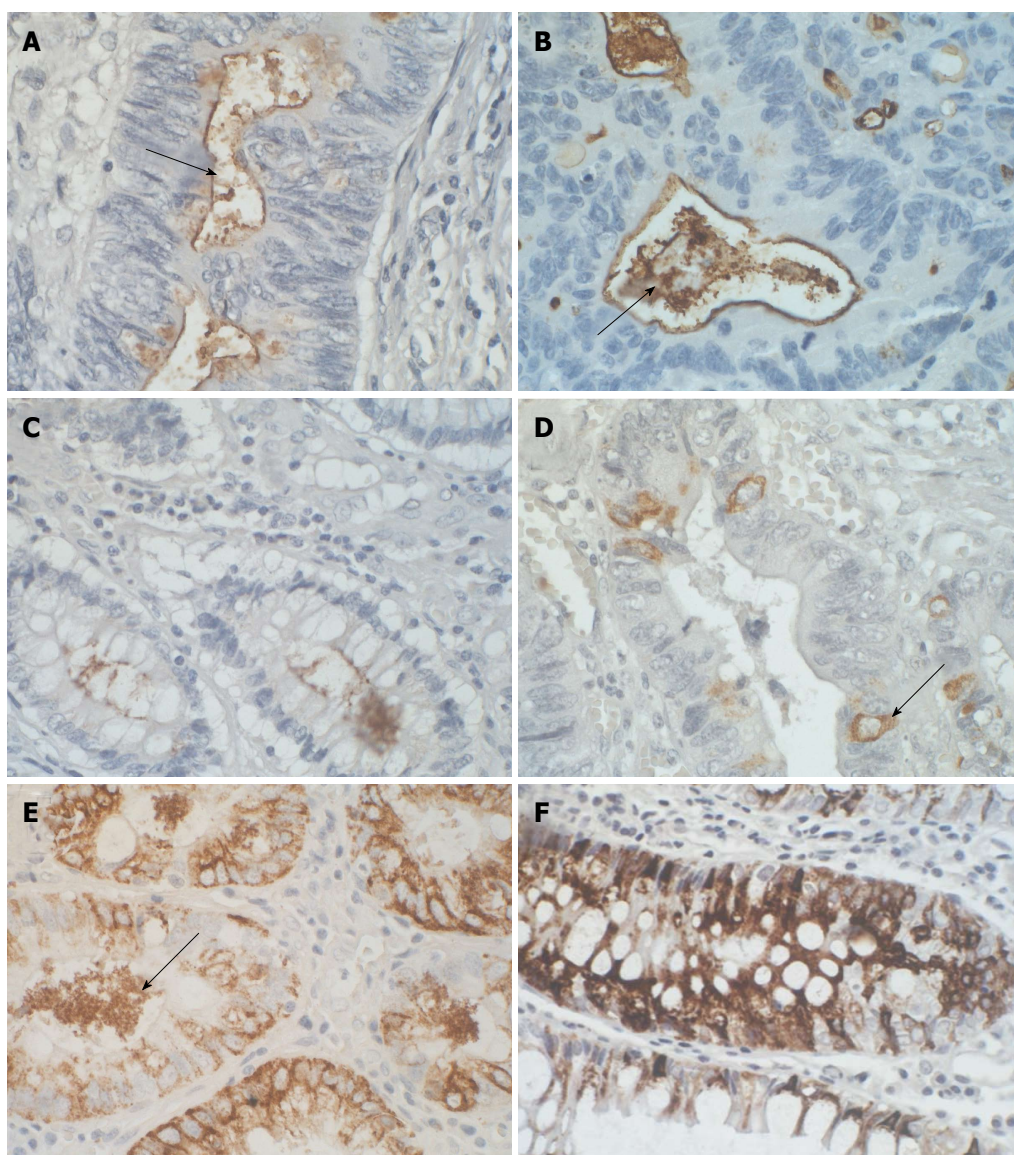
A significantly higher expression of MUC2 in mucinous CRC ( $10.97\% \pm 11.17\%$  of IHC reaction), compared to the rest of the CRC ( $2.40\% \pm 2.00\%$ ), was shown ( $P = 0.018$ ). No such differences were observed with MUC1 expression (Figure 2). No significant differences could be disclosed in the expression of both mucins from one hand and tumor size, the anatomical location of the CRC, histologic grade (G2 vs G3) and in patients with N0 and N1 in the TNM classification system (data not shown).

In the case of MUC2 expression, significantly higher expression of this mucins in colorectal tumors in Stage C was shown ( $5.54\% \pm 7.57\%$ ), compared with Stage B ( $2.12\% \pm 2.64\%$ ) ( $P = 0.044$ ) (Figure 3). The analysis of mucins expression in tumors of Stage B2 and C2 on Astler and Collier scale, confirmed these results, although only a borderline statistical significance ( $P = 0.066$ ) was obtained for MUC2 expression (data not shown).

#### **MUC1 and MUC2 transcript vs protein expression**

High positive Spearman's correlation was observed in patients affected by CRC, between mutual expression of both analyzed mucin transcripts ( $r = 0.602$ ;  $P < 0.05$ ), but not between protein expression itself ( $r = 0.046$ ).





**Figure 1** Immunohistochemical illustrations of colorectal carcinoma and control colon with mucin 1 and mucin 2 positive expression. A: Representative IHC expression of MUC1 in luminal surface epithelium (arrow) of tumor-changed colon crypt; B: Membranous and extracellular pattern (arrow) of MUC1 expression in neoplastic cells lining the glandular structures of CRC; C: Representative image of MUC1 membranous localization in normal colon crypts; D: Cytoplasmic expression of MUC2 in scattered epithelial cells of the tumor-changed colon crypt (arrow); E: IHC intense reaction of MUC2 expression in the cytoplasm of numerous cancer cells and/or localized in the lumen of the colon crypts (arrow); F: Cytoplasmic expression of MUC2 in majority of goblet cells in normal colon epithelium. Sections were counterstained with hematoxylin. Objective  $\times 40$ . MUC1: Mucin 1; MUC2: Mucin 2; IHC: Immunohistochemical; CRC: Colorectal cancer.

(Table 4). Additionally, in CRC tumor tissues, borderline positive Spearman's correlation, between mRNA and protein expression of MUC1 ( $r = 0.405$ ;  $P = 0.055$ ). MUC2 didn't show a statistically significant correlation between mRNA and protein expression (Table 4).

#### **MUC1 and MUC2 expression (mRNA and proteins) and clinical data**

Negative correlation between MUC1 mRNA expression and the age of patients was observed ( $r = -0.481$ ;  $P < 0.05$ ). Furthermore, positive correlations considered the expression of both mucins (MUC1 and MUC2) and the blood leukocyte count ( $r = 0.465$  and  $r = 0.474$  respectively;  $P < 0.05$  in both cases). Additionally, the expression of MUC1 protein was positively correlated

with thrombocyte numbers in patients affected by CRC ( $r = 0.474$ ;  $P < 0.05$ ) (Table 5).

Mean survival time of patients affected by CRC was  $52 \pm 3$  mo. The Kaplan-Meier analysis shows that neither MUC1, nor MUC2 apomucins expression were significantly associated with survival probability in patients with CRC (Figure 4A and B). Survival curves of 34 patients with CRC showed that also expression of mRNA for both mucins in tissue samples was not associated with the prognosis of CRC (data not shown).

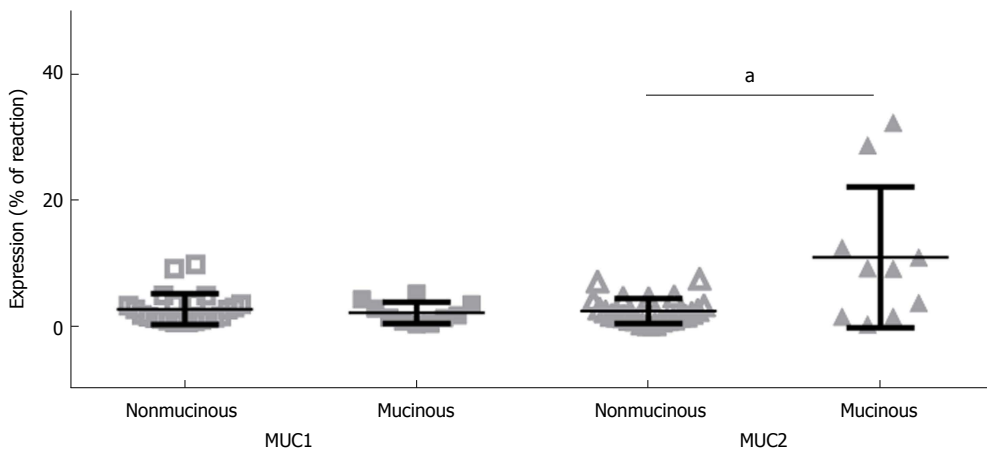
#### **Ki-67 proliferating antigen and p53 immunoexpression**

The positive expression of Ki-67 proliferating antigen was detected in 28/34 (82%) of CRC tissue samples. Additionally, a significantly higher expression of Ki-67 in

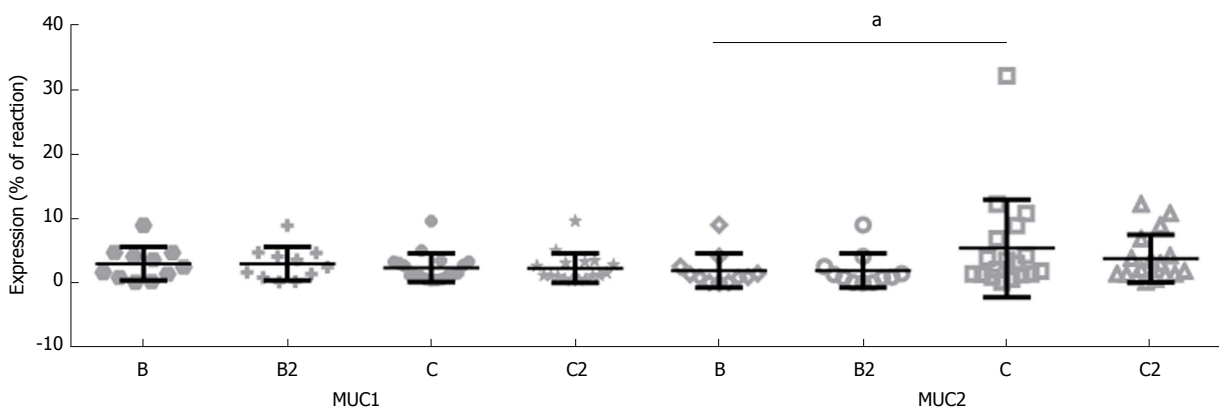
**Table 4** Values of Spearman's coefficient for correlation between both mucins (mRNA, protein) and Ki-67 and/or p53 protein expressions in colorectal carcinoma samples

|           | MUC1               | MUC2                | mRNA MUC1          | mRNA MUC2          | Ki-67               | p53                 |
|-----------|--------------------|---------------------|--------------------|--------------------|---------------------|---------------------|
| MUC1      | -                  | 0.046               | 0.405 <sup>a</sup> | 0.199              | 0.015               | -0.106              |
| MUC2      | 0.046              | -                   | 0.457              | 0.126              | -0.428 <sup>1</sup> | -0.389 <sup>1</sup> |
| mRNA MUC1 | 0.405 <sup>a</sup> | 0.457               | -                  | 0.602 <sup>1</sup> | -0.121              | -0.215              |
| mRNA MUC2 | 0.199              | 0.126               | 0.602 <sup>1</sup> | -                  | 0.033               | -0.145              |
| Ki-67     | 0.015              | -0.428 <sup>1</sup> | -0.121             | 0.033              | -                   | 0.602 <sup>1</sup>  |
| p53       | -0.106             | -0.389 <sup>1</sup> | -0.215             | -0.145             | 0.602 <sup>1</sup>  | -                   |

<sup>1</sup>Indicate values of  $r$  coefficient for which  $P < 0.05$ ; <sup>a</sup> $P = 0.055$ . MUC1: Mucin 1; MUC2: Mucin 2.



**Figure 2** Comparative immunoexpression of mucin 1 and mucin 2 in nonmucinous and mucinous subtypes of colorectal carcinoma. Mean  $\pm$  SD. <sup>a</sup> $P$  (level of significance) value  $< 0.05$ . MUC1: Mucin 1; MUC2: Mucin 2.



**Figure 3** Tissue expression of mucin 1 and mucin 2 in colorectal carcinoma as related to Dukes and Astler and Collier staging system. Mean  $\pm$  SD. B, C: Dukes staging system; B2, C2: Astler and Collier staging system. <sup>a</sup> $P$  (level of significance) value  $< 0.05$ . MUC1: Mucin 1; MUC2: Mucin 2.

CRC as compared with control was demonstrated ( $P < 0.001$ ) (data not shown).

Only the nuclear location of Ki-67 within different percentages of immunopositive tumor cells was observed (Figure 5A and B). The Ki-67 antigen expression in the control samples was evident mainly in the individual basally located nuclei of the goblet cells lining the unaltered intestinal crypts (Figure 5C).

Positive expression of p53 was demonstrated in 19/34 (56%) patients. Similar to Ki-67 antigen, only the nuclear location was observed and, as a rule, a very

intense IHC reaction concerning the majority of polymorphic cell nuclei in the evaluated samples (Figure 5D). 44% of CRC patients did not show the presence of the protein in tumor samples (Figure 5E). In the healthy colorectal samples (control), p53 expression was not detected in any specimen (Figure 5F).

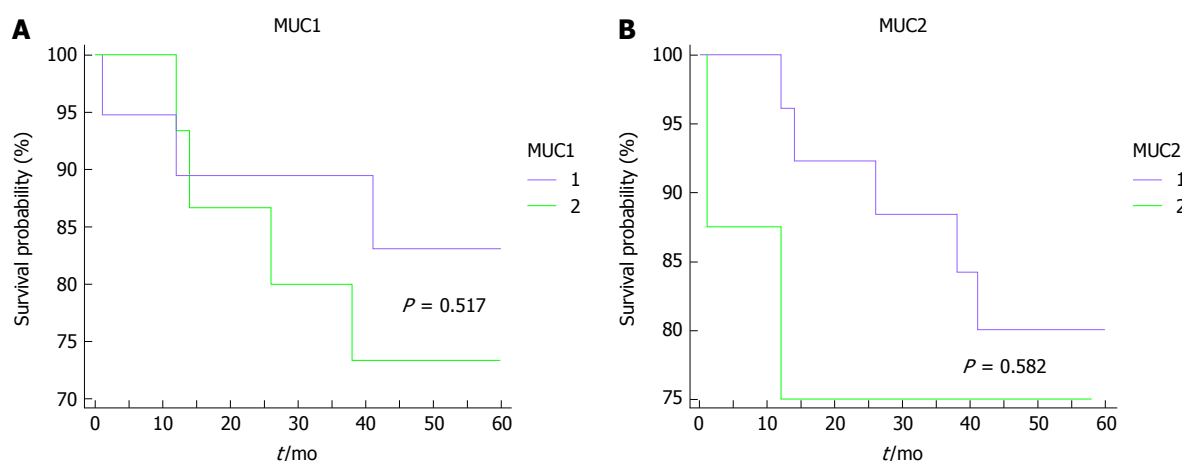
#### **MUC1 and MUC2 expression (mRNA and proteins) vs Ki-67 and p53 expression**

A significant, relatively high, negative Spearman's correlation, between the expression of MUC2 apomucin and

**Table 5** Values of Spearman's coefficient for correlation between mucins expression (mRNA/protein) in colorectal carcinoma and selected clinical data

|           | Age (yr)            | Hemoglobin (g/dL) | WBC ( $\times 10^9/L$ ) | Thrombocytes (g/L) | Glucose (mg/dL) |
|-----------|---------------------|-------------------|-------------------------|--------------------|-----------------|
| MUC1      | -0.322              | -0.175            | 0.067                   | 0.474 <sup>1</sup> | -0.277          |
| MUC2      | 0.053               | -0.123            | -0.097                  | -0.085             | -0.346          |
| mRNA MUC1 | -0.481 <sup>1</sup> | 0.189             | 0.465 <sup>1</sup>      | 0.203              | -0.325          |
| mRNA MUC2 | -0.412              | -0.098            | 0.474 <sup>1</sup>      | -0.145             | -0.306          |

<sup>1</sup>Indicate values of  $r$  coefficient for which  $P < 0.05$ . WBC: White blood cell; MUC1: Mucin 1; MUC2: Mucin 2.



**Figure 4** Kaplan-Meier survival curves for colorectal carcinoma patients, as related to tissue expression of mucin 1 and mucin 2 showing that expression of both mucins in tissue samples are not associated with survival time. A: Kaplan Meier survival curve related to tissue expression of MUC1; B: Kaplan Meier survival curve related to tissue expression of MUC2. 1: Under mean tissue expression; 2: Above mean tissue expression. MUC1: Mucin 1; MUC2: Mucin 2.

Ki-67 ( $r = -0.428$ ;  $P < 0.05$ ) was observed (Figure 6). In contrast, relatively weak, negative correlation between MUC2 protein and p53 expression ( $r = -0.389$ ;  $P < 0.05$ ), was shown in CRC tissues (data not shown). Additionally, a high positive correlation was observed for Ki-67 antigen and p53 expression in study group ( $r = 0.603$ ;  $P < 0.05$ ) (data not shown).

## DISCUSSION

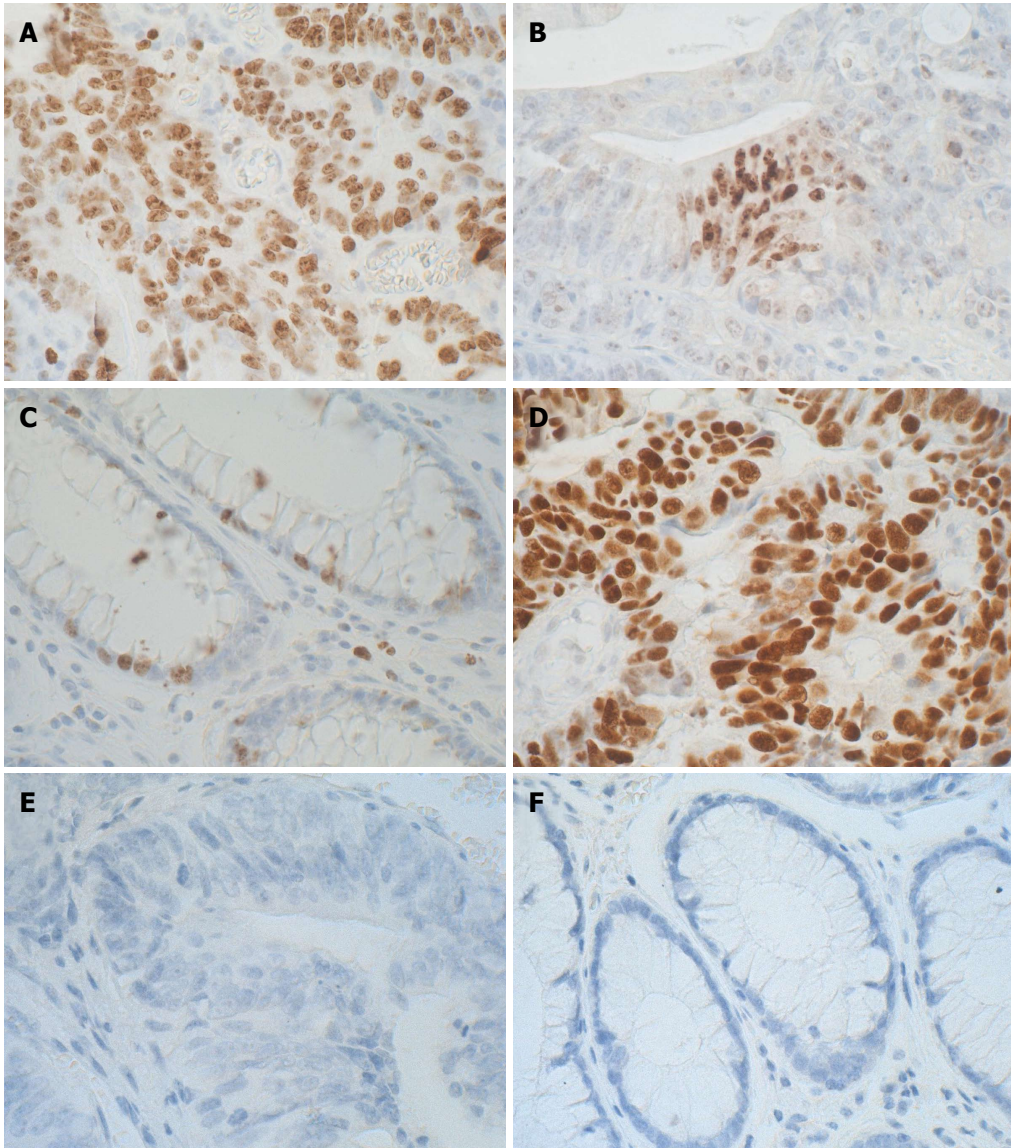
Some discrepancies are present between the results of MUC1 expression detection, in healthy colon and rectum, with the use of immunohistochemistry. Some scientific publications, notably the recent ones, document lack of MUC1 expression in control large intestine in adults<sup>[18-20]</sup>, or emphasize low detectability (10%) of this apomucin<sup>[36]</sup>.

In this study, positive MUC1 expression (mRNA, protein) was observed in almost all of the control samples of large intestinal tissue. These results are coherent with the findings of other authors, conducted with the use of light and electron microscopy<sup>[37,38]</sup>. Descriptions of clear, membranous expression of MUC1, on the luminal surface of glandular cells of normal colon epithelium, are available also in interactive databases<sup>[39]</sup>. Therefore, our own research using IHC technique confirms both, detectability and evident membranous expression of this mucin, in tissues of healthy large intestine.

In neoplastic CRC tissues collected in this study,

detectability of MUC1 expression *via* IHC was 100%, being much higher than those achieved by other authors, which note it from below 20%<sup>[40]</sup>, through 32%-40%<sup>[14,18,41]</sup>, approximately 55%<sup>[20]</sup>, to 70%-80%<sup>[29]</sup>. According to some publications, MUC1 expression was more commonly detected in CRC patients with lymph node metastases discovered during surgical procedures, than those without such metastases (84.2% vs 34.6%)<sup>[20]</sup>, which is not confirmed by the current study. However, our results are similar to those obtained in CRC tissue microarrays, in which the authors also did not observe correlation between MUC1 expression and histologic grade, stage, vascular invasion, or cancer type<sup>[14,30]</sup>. The results of research by Matsuda *et al.*<sup>[41]</sup>, concerning more common expression of MUC1 in CRC of more severe histologic stages, were not confirmed in our studies. However, similarly to the authors<sup>[41]</sup>, we have also not found any correlation between MUC1 and p53 expression. As in our studies, Kesari *et al.*<sup>[19]</sup> did not observe differences in MUC1 expression depending on the histologic stage, but a higher incidence of expression of this mucin was described in G2, than in G1 of this cancer (55% vs 11%)<sup>[19]</sup>. The positive relationship between MUC1 expression and histologic grade and stage can also be found in other publications, with the appreciation of this expression as a high risk factor of death in Caucasian population (HR: 2.03;  $P = 0.038$ )<sup>[29,37]</sup>. The co-expression of MUC1 and p53 was a bad prognostic factor for the overall survival (OS) of these patients<sup>[29]</sup>. Our own





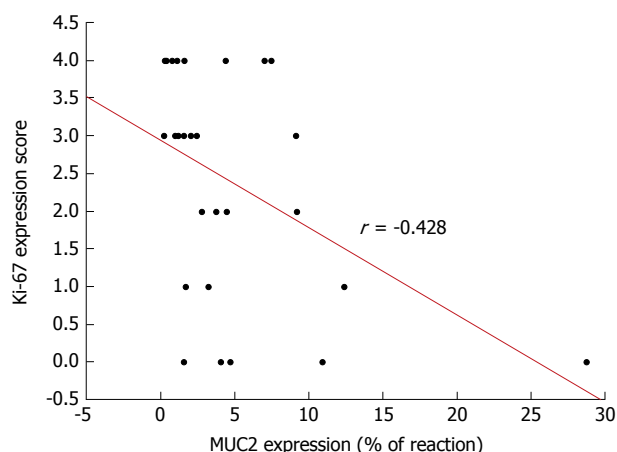
**Figure 5** Immunohistochemical illustrations of colorectal carcinoma and control colon with Ki-67 antigen and p53 expression. A: Representative IHC expression of Ki-67 proliferating antigen in the majority of tumor cell nuclei; B: An intense nuclear pattern of Ki-67 expression in focally located tumor cells; C: Representative image of Ki-67 proliferating antigen immunoexpression in the individual basally located nuclei of the goblet cells lining the unaltered intestinal crypts; D: A pronounced p53 nuclear pattern of IHC reaction in glandular structures of CRC; E: Negative IHC reaction for p53 in tumor of other CRC patient; F: Negative IHC reaction for p53 in normal colon. Sections were counterstained with hematoxylin. Objective  $\times 40$ . IHC: Immunohistochemical; CRC: Colorectal cancer.

research cannot confirm the above observations, as well as the results of other authors, where MUC1 expression was shown to be an independent marker of prognosis (HR: 1.339, 95%CI: 1.002-1.790;  $P = 0.048$ )<sup>[14]</sup>. The lack of dependence between MUC1 expression and histologic grade or stage, in the current study probably results from the very homogenous group of patients with CRC in the range of histologically assessed parameters [68% with grade 2; 56% with stage C (C1-C3)/D in Dukes/Astler and Collier scale; 85% with stage III and IV in TNM classification system]. Furthermore, it should be stressed, that the results of multiple authors are mainly based on the analysis of the detection (incidence) of MUC1 expression, rather than a reliable quantitative assessment<sup>[19,29]</sup>. In several works, MUC1 expression was admittedly evaluated, using semi-quantitative me-

thods<sup>[20,30,36]</sup>, but some of them did not have control groups<sup>[14,19,30]</sup>. There are publications that intensify the IHC reaction, and introduce the division into the so-called high, low and negative MUC1 expression, occurring in a different percentage of patients (12%, 52%, 36%, respectively)<sup>[30]</sup>. Hence, the overexpression of the MUC1 protein was observed in varying percentages in different patients, from 12%<sup>[30]</sup> to approximately 40%<sup>[19]</sup>.

Most of the cited researchers, in their studies, used monoclonal, primary Ma695 antibody (Novocastra), in 1:100 dilution<sup>[18,19,30,40]</sup>. In the current study, antibodies from the same company have been used, also in 1:100 dilution. However, we have chosen another clone (Ma552). Furthermore, the use of a reliable, repeatable method of quantitative evaluation of IHC expression (HSV filter program), may explain the discrepancies obtained in the





**Figure 6** Spearman's correlation between the expression of mucin 2 protein and of Ki-67 proliferating antigen in colorectal carcinoma. MUC2: Mucin 2.

study results, at least partially, including the increased MUC1 expression in both control, and cancerous large intestine, presented in our study.

Current studies, employing the RT-qPCR method, have been shown a significantly higher mean expression of MUC1 mRNA in CRC, compared to the healthy tissue. This expression showed borderline correlation ( $P = 0.055$ ) with MUC1 protein expression. It is difficult to relate this result to the literature data.

The results of correlation analysis, between the MUC1 expression (mRNA, protein) and the available patient clinical data, are not as spectacular as those obtained by other authors. In the case of MUC1, a negative correlation is shown between the mRNA (but not the protein itself) expression and the age of the CRC affected patients. The data on that specific relation was not found in literature. In addition, we have observed positive correlations between the MUC1 mRNA and the number of leukocytes, while the expression of the MUC1 apomucin itself, correlated with the number of platelets in the CRC. There are also no exact references to these results in literature. However, in mouse model, it was shown that carcinoma mucins (fragments from human colonic adenocarcinoma LS180 cells) initiate thrombosis through adhesion-dependent, reciprocal activation of neutrophils and platelets. These studies provide insights into mucin-dependent, thrombin-independent thrombosis in patients with Trousseau syndrome<sup>[42]</sup>.

Similar to MUC1 expression, the detectability of MUC2 (mRNA, protein), obtained in the current, study was higher, than that presented in the literature data, which cite approximately 30%<sup>[14]</sup>, approximately 50%-64%<sup>[20,30,43]</sup> and 92% of positive CRC cases<sup>[44]</sup>. Some sources document a higher incidence of MUC2 expression detection (72%-100%), only in the case of mucinous CRC subtypes<sup>[29,40]</sup>. The production of MUC2 mRNA in the healthy colorectal tissues is documented by numerous researchers<sup>[18,45,46]</sup>, although some of the authors describe it in just 20% of the control tissues<sup>[44]</sup>.

The cytoplasmic pattern of MUC2 expression, de-

monstrated in the present work, confirms previous observations in the healthy and cancer-altered large intestine<sup>[39,46,47]</sup>. We have described similar amounts of MUC2 mRNA in CRC and control, but a lower expression of the MUC2 protein in patients with CRC, compared to control. Confronting this with literature, the results of the IHC study are consistent with those obtained by many researchers<sup>[14-20,29,41,43,48]</sup>, while in the case of MUC2 transcripts detection, few works record reduced expression of MUC2 mRNA in the CRC, compared to the normal tissue<sup>[45]</sup>. In the current work, higher expression of MUC2 in the mucinous CRC, as compared with nonmucinous subtypes of cancer, has been shown, which also confirms the results of other authors' research<sup>[24,47,49]</sup>.

In CRC patients gathered in this work, a higher expression of MUC2 was also observed in the more advanced histologic stages of the tumor. In addition, there have been significant negative correlations between MUC2 with Ki-67 and p53 expressions. This could indicate a significant relation between the decrease in the expression of this glycoprotein in the course of colon carcinogenesis, and the pro-proliferative activity (Ki-67), or deregulation of the tumor suppressive P53 signaling. This result is confirmed by the research of other authors<sup>[50,51]</sup>. However, similarly to MUC1, there was no significant correlation between MUC2 expression and histologic grade, size, or location of the tumor, which is also consistent with the literature data<sup>[43,52]</sup>.

In the current study, it was also not possible to find statistically significant relationships between mucin expression (mRNA, protein) and survival of patients with CRC. In the 5-year period evaluated, seven people died of cancer (21%), the average survival was 52 mo from the time of surgery. The small number of patients analyzed, including the deceased, did not allow to draw binding conclusions on the predictive role of MUC1 and MUC2 tissue expression, in the CRC patients of the Greater Poland Region.

Research by other authors points to a link between MUC1 overexpression and poorer survival, especially in mucinous tumors. These authors prove, that higher frequency of MUC1 immunoreactivity in the mucinous subtype of CRC was independently related to greater rate of cancer death in colorectal patients<sup>[26]</sup>. In the case of MUC2, however, other studies have also shown important correlations between MUC2 expression reduction/loss, shorter survival time (OS), shorter progression-free/disease-free (PFS) in patients with stage II and III colorectal carcinomas<sup>[30,53]</sup> and longer disease-free (DFS) and disease-specific survival (DSS) in patients with positive MUC2 expression. The loss of expression of this mucin was correlated with the recurrence of cancer<sup>[52]</sup>. In some studies, the relationship between MUC2 expression and survival was not spectacular, but only borderline, and more often concerned well-to-moderately differentiated adenocarcinomas [ $P = 0.064$  for recurrence/metastasis-free survival (RFS) and  $P = 0.172$  for OS] but not for poorly differentiated adenocarcinomas<sup>[43]</sup>.

Although the current study is based on a relatively small group of patients ( $n = 34$ ), with the predominance of nonmucinous subtype of CRC, it can be assumed, that the expression of both mucins (MUC1 and MUC2), at the level of mRNA and protein, occurs in a normal and tumor-altered colon. Lower tissue expression of MUC2 in CRC, as compared with control, correlates with increased cellular proliferation and could become a marker of cancer progression. The intensity of MUC2 expression allows to differentiate mucinous and nonmucinous CRC subtypes.

The clinical limitations of the current study can be summarized as follows: (1) Most likely due to the homogeneous study group in the range of histologically assessed parameters (68% patients with G2 and 85% with stage III and IV in TNM classification), not all differences in MUC1 and MUC2 expression or correlations with clinical data have reached statistical significance. And (2) The small number of deceased patients ( $n = 7$ ) analyzed in the current study, did not allow to draw binding conclusions on the predictive role of MUC1 and MUC2 tissue expression for the survival time of patients with CRC of the Greater Poland Region.

Future study is required and a larger number of patients should be evaluated to confirm our findings. Better characterization of the role of mucins in molecular mechanisms in colorectal carcinogenesis requires further testing, also on an *in vitro* model.

In conclusion, a combination of tissue overexpression of MUC1, reduced MUC2 expression, and high ratio of MUC1/MUC2 is a factor of poor prognosis in CRC patients. MUC2 tissue expression allows to differentiate mucinous and nonmucinous CRC subtypes.

## ARTICLE HIGHLIGHTS

### Research background

In Poland, colorectal carcinoma (CRC) is the second most common cancer in men and woman, with the third leading causes of cancer deaths in Greater Poland Region. Altered mucin expression is correlated with the prognosis of this cancer. *In vivo* as well as *in vitro* studies on the expression of mucins may have also therapeutic implications.

### Research motivation

The role of mucin expression at various stages of the colon carcinogenesis is incomplete. The prognostic role of mucins in the mucinous subtypes of CRC is poorly known. Research into the role of mucins in pathogenesis and CRC clinical studies (especially in mucinous subtypes) are also current topics from a methodological point of view. The lack of standardized methods of quantitative evaluation of mucins expression (especially at tissue level) and/or frequent lack of control groups, are a great difficulty in comparative analysis.

### Research objectives

Current research determines tissue expression (mRNA, protein) of membrane-bound mucin [mucin 1 (MUC1)] and secretory mucin [mucin 2 (MUC2)] in healthy and colorectal cancer tissue samples and evaluates the relationship between tissue expression of both mucins and selected clinicopathological data of the patients with CRC.

### Research methods

The research on tissue expression of two types of mucins (MUC1 and MUC2)

in cancerous and normal colorectal tissue samples was performed using real-time quantitative polymerase chain reaction (RT-qPCR) to evaluate expression of transcripts, immunohistochemistry (IHC) for demonstrating apomucins localization, and the morphometric analysis of intensity of IHC reaction using modern HSV filter software.

### Research results

Significantly higher expression of the MUC1 mRNA in the CRC, while MUC2 transcript expression was comparable with the control colorectal samples. Using immunohistochemistry, we observed lower MUC2 protein as compared to control tissue. MUC2 protein expression correlated negatively with cellular proliferation (Ki-67 antigen expression) and expression of mutated form of p53. In neoplastic tissue of CRC it was observed also higher MUC1/MUC2 ratio as compared with healthy colorectal tissue. Higher expression of MUC2 was a feature of mucinous CRC subtypes, and characterized higher histological stage of tumors. Future study is required to explain molecular mechanisms of CRC carcinogenesis including mucins and TP53 pathway.

### Research conclusions

Our study confirmed that the colorectal carcinogenesis is closely related to overexpression of MUC1 and the decline in MUC2 expression. The use of increasingly repetitive and reliable method for the quantitative evaluation of mucins expression may prove useful to evaluate different patterns of IHC reaction (membranous, cytoplasmic, etc.) and can be useful in various subtype of colorectal cancer, as our research shows (mucinous vs nonmucinous CRC). Both the microscopic demonstration of evident MUC1 expression, especially in healthy colorectal tissue (control), and morphometric quantitative evaluation (Filter HSV program) of membranous (MUC1) and secreted (MUC2) expression is the novelty of the present work. The quantitative method used in the current study, can be used for further comparative research and to evaluate tissue expression of other types of mucins in CRC. The use of quantitative methods in immunocytochemistry can improve the detection of tissue markers in CRC and assess their true value in daily medical practice.

### Research perspectives

This study proposed, that the examination of the tissue expression of MUC1 and MUC2 (mRNA, protein), should use modern methods of quantitative assessment of transcripts (e.g. RT-qPCR), and more reliable morphometric methods (e.g. HSV filter software). Only these methods could improve the diagnostic/prognostic usefulness of mucins as tissue biomarkers in CRC patients. A better explanation of molecular mechanisms in the colorectal carcinogenesis with mucin involvement requires further testing, also on an *in vitro* model. These results could be the basis for further studies to understand the carcinogenesis of colorectal cancer.

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## Basic Study

# Mechanism of combined use of vitamin D and puerarin in anti-hepatic fibrosis by regulating the Wnt/ $\beta$ -catenin signalling pathway

Gan-Rong Huang, Si-Jun Wei, Yan-Qiang Huang, Wei Xing, Lu-Yao Wang, Ling-Ling Liang

Gan-Rong Huang, Yan-Qiang Huang, Wei Xing, Lu-Yao Wang, Ling-Ling Liang, Youjiang Medical University for Nationalities, School of Basic Medical Sciences, Baise 533000, Guangxi Zhuang Autonomous Region, China

Si-Jun Wei, Affiliated Hospital of Youjiang Medical University for Nationalities, Baise 533000, Guangxi Zhuang Autonomous Region, China

ORCID number: Gan-Rong Huang (0000-0001-5580-8689); Si-Jun Wei (0000-0001-6948-2747); Yan-Qiang Huang (0000-0002-2121-8258); Wei Xing (0000-0001-5640-1151); Lu-Yao Wang (0000-0001-5545-0170); Ling-Ling Liang (0000-0002-1498-2370).

**Author contributions:** Huang GR and Wei SJ contributed equally to this work; Huang YQ, Wang LY and Liang LL performed the majority of the experiments; Xing W performed the experimental design, writing and analysis.

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**Correspondence to:** Wei Xing, MD, Professor, Youjiang Medical University for Nationalities, School of Basic Medical Sciences, 98 Urban-Rural Road, Baise 533000, Guangxi Zhuang Autonomous Region, China. 116895009@qq.com  
Telephone: 18907767511

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## Abstract

### AIM

To reveal the protective mechanism of the combined use of vitamin D and puerarin in the progression of hepatic fibrosis induced by carbon tetrachloride (CCl<sub>4</sub>).

### METHODS

Eight-week-old male Wistar rats were randomly divided into a normal control group (C group), a CCl<sub>4</sub> group (CCl<sub>4</sub> group), a vitamin D group (V group), a puerarin group (P group), and a combined group of vitamin D and puerarin (V + P group), each of which contained ten rats. In this way, we built a rat model of CCl<sub>4</sub>-induced hepatic fibrosis with intervention by vitamin D, puerarin, or a combination of the two. After eight weeks, the mice were sacrificed to collect serum and liver specimens. Blood was collected to detect the hyaluronic acid (HA). We also measured hydroxyproline (Hyp) and prepared paraffin sections of liver. After Sirius red staining, the liver specimens were observed under a microscope. RT-PCR and western blot analysis were adopted to detect the mRNA and the protein

levels of Collagen I, Collagen III, Wnt1, and  $\beta$ -catenin in the liver tissues, respectively.

## RESULTS

Hepatic fibrosis was observed in the CCl<sub>4</sub> group. In comparison, hepatic fibrosis was attenuated in the V, P, and V + P groups: the HA level in blood and the Hyp level in liver were reduced, and the mRNA levels of Collagen I, Collagen III, Wnt, and  $\beta$ -catenin in liver were also decreased, as well as the protein levels of Wnt1 and  $\beta$ -catenin. Among these groups, the V + P group demonstrated the greatest amelioration of hepatic fibrosis.

## CONCLUSION

The combined application of vitamin D and puerarin is capable of alleviating CCl<sub>4</sub>-induced hepatic fibrosis of rats. As to the mechanism, it is probably because the combined use is able to silence the Wnt1/ $\beta$ -catenin pathway, suppress the activation of hepatic stellate cells, and reduce the secretion of collagen fibers, therefore improving the anti-hepatic fibrosis effect.

**Key words:** Carbon tetrachloride; Hepatic fibrosis; Vitamin D; Puerarin; Wnt/ $\beta$ -catenin

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**Core tip:** The proliferation of hepatic stellate cells (HSCs) is associated with hepatic fibrosis. The activated HSCs, as well as Wnt1 and  $\beta$ -catenin, have become important targets in anti-hepatic fibrosis therapy. This research investigated the protective effect of the combined use of vitamin D and puerarin against CCl<sub>4</sub>-induced hepatic fibrosis in rats. The protective effect of the combined use of vitamin D and puerarin in the progression of hepatic fibrosis is closely associated with the function of silencing the Wnt1/ $\beta$ -catenin pathway, suppressing the activation of HSCs, and decreasing the secretion of collagen fibers, which provided a useful reference for those in clinical practice.

Huang GR, Wei SJ, Huang YQ, Xing W, Wang LY, Liang LL. Mechanism of combined use of vitamin D and puerarin in anti-hepatic fibrosis by regulating the Wnt/ $\beta$ -catenin signalling pathway. *World J Gastroenterol* 2018; 24(36): 4178-4185 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i36/4178.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i36.4178>

## INTRODUCTION

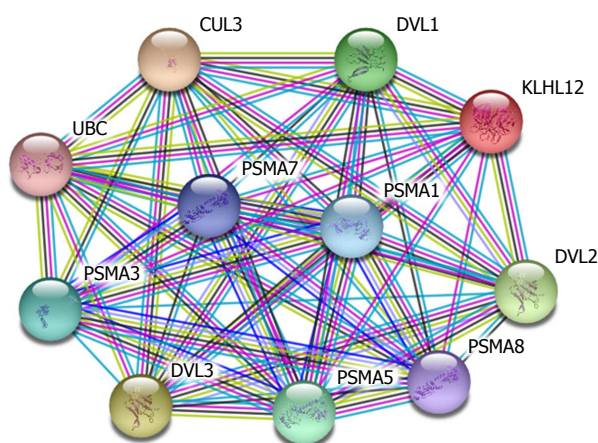
Hepatic fibrosis is the change of pathological structures due to all kinds of chronic liver diseases. Essentially, it occurs because extracellular matrix (ECM) synthesized by hepatic stellate cells (HSCs) under the actions of various pathogenic factors substantially increases,

exceeding the degradation ability of the liver itself. The long-term accumulation of ECM will lead to hepatic fibrosis and thus cause liver cirrhosis<sup>[1,2]</sup>. In recent years, it has been proven that the  $\beta$ -catenin protein plays a critical role in the occurrence and development of hepatic fibrosis<sup>[3,4]</sup>. Previous studies revealed that the more serious the hepatic fibrosis, the higher the expression of  $\beta$ -catenin in liver tissues compared with normal liver tissues, and silencing  $\beta$ -catenin is able to suppress the secretion of collagen and the proliferation of HSCs and mediate cell apoptosis.  $\beta$ -catenin participates in many signalling pathways, among which the Wnt/ $\beta$ -catenin pathway is the most common along which  $\beta$ -catenin plays its role. Apart from this,  $\beta$ -catenin also participates in alternative pathways including E-cadherin, NF- $\kappa$ B, and TGF- $\beta$ <sup>[5-7]</sup>. Therefore,  $\beta$ -catenin is key to the intersection of multiple signalling pathways. Existing experiments revealed that HSC-T6 cell membranes and cytoplasm with activated phenotypes show  $\beta$ -catenin expression, and  $\beta$ -catenin expression is also observed in nuclei. This indicates that the Wnt/ $\beta$ -catenin signalling pathway is activated in activated HSCs. The expression of  $\alpha$ -SMA in HSC-T6 cells was down-regulated by blocking the transduction of the Wnt/ $\beta$ -catenin signalling pathway, and the expression of types I and III collagen (Collagen I and Collagen III) was also significantly down-regulated. The analysis based on the String database finds that DVL1, DVL2, and DVL3 play their functions in signal transduction pathways mediated by multiple Wnt genes. They are regulatory factors for the Wnt signalling pathway and ER-to-Golgi transport and participate in the ER-to-Golgi transport, thus KLHL12 plays a critical role in the export of collagen, as shown in Figure 1. This implies that the Wnt/ $\beta$ -catenin signalling pathway is closely associated with the activation of HSCs.

Carbon tetrachloride (CCl<sub>4</sub>) is one of the classical poisons used in establishing hepatic fibrosis models and is therefore widely used in fundamental research<sup>[8]</sup>. Previous research has proven *in vivo* that vitamin D is able to alleviate hepatic fibrosis, and *in vitro* experiments confirmed that vitamin D can reduce the secretion of collagen fibers of HSCs<sup>[9,10]</sup>. There are also evidences from existing research that puerarin is capable of attenuating hepatic fibrosis, which is probably associated with the inhibition of the activation of HSCs by blocking the TNF- $\alpha$  signalling pathway<sup>[11,12]</sup>. A preliminary study by the present research team has confirmed that the combined use of vitamin D and puerarin is able to enhance the anti-hepatic fibrosis effect; however, the related mechanism has not been revealed. Therefore, the current research investigated the effects of vitamin D combined with puerarin on the expression of key factors, including Wnt1,  $\beta$ -catenin, Collagen I, and Collagen III, in the Wnt/ $\beta$ -catenin signalling pathway based on the aforementioned preliminary study. By doing so, we attempted to further clarify the mechanism of the combined use of vitamin D

**Table 1** Designations and sequences of primers

| Designation (mice) | Sequence (5'-3')  |
|--------------------|---|
| GAPDH              | F: GGCATCCTGACCCCTCAAGTA<br>R: GGGGTGTGAACCTCTCAAA          |
| Collagen I         | F: GGACACTACTGGATCGACCTAAC<br>R: CTCACCTGTCTCCATGTTGCA      |
| Collagen III       | F: CTACCTTGCTCAGTCCTATGAGTCTAGA<br>R: TCCCGAGTCGCAGACACATAT |
| Wnt1               | F: GAAACCGCCGCTGGAACCT<br>R: CCGTCCTCGTATATGTGAAG           |
| $\beta$ -catenin   | F: ACC TCC CAAGTC CTG TAT<br>R: CCT GGT CCT CGT CAT TTA     |

**Figure 1** Interaction between WNT-related genes and collagen-related genes.

and puerarin in anti-hepatic fibrosis.

## MATERIALS AND METHODS

### Experimental materials

Fifty clean-grade healthy male Wistar rats (with a body mass of about 200 g) of similar age were provided by the Animal Center of Youjiang Medical College for Nationalities. Analytically pure CCl<sub>4</sub> was purchased from Sinopharm Chemical Reagent Co., Ltd, and vitamin D and puerarin were purchased from Sigma. In addition, corn oil, Sirius red staining solution, and real-time fluorescence quantitative PCR kits were purchased from Wako Pure Chemical Industries, Ltd, Beijing Leagene Biotech Co., Ltd, and Roche (Switzerland), respectively. Wnt1 and  $\beta$ -catenin antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, United States). Table 1 shows the designations and sequences of primers.

### Experimental methods

Fifty Wistar rats were randomly divided into five groups. Rats in the CCl<sub>4</sub> group were administered 3 mL/kg of corn oil solution containing 45% CCl<sub>4</sub> through intraperitoneal injection twice a week. The normal control group (C group) was administered the same dose of normal saline through intraperitoneal injection twice a week. The V, P, and V + P groups were the drug intervention

groups. On the basis of the models established using corn oil solution containing CCl<sub>4</sub>, mice in the three groups received 2  $\mu$ g/kg of vitamin D, 0.4 g/kg of puerarin, and a combination of D vitamin (2  $\mu$ g/kg) and puerarin (0.4 g/kg) through intragastric administration twice a week. The mice were subjected to fasting for one hour before each administration of treatment. The C group received the same quantity of normal saline through intragastric administration. After eight weeks, the mice were fasted overnight the day before the end of the experiments and sacrificed the next day. Whole blood was collected and stood for 20 min at room temperature. Then, the blood was centrifuged at 5000 rpm for 15 min. Thereafter, the supernatant and then the serum were obtained, followed by the detection of hyaluronic acid (HA) in blood using the aforementioned kit. After collecting the liver of the rats, the kit was used to detect hydroxyproline (Hyp) in the liver. Part of the liver was fixed in a paraformaldehyde solution, and paraffin sections were prepared. After Sirius red staining, liver tissue slices were placed under the microscope to observe their pathological changes and degree of fibrosis. Moreover, real-time fluorescence quantitative PCR was applied to detect the mRNA levels of Collagen I, Collagen III, Wnt1, and  $\beta$ -catenin, and a western blot assay was used to measure the protein levels of Wnt1 and  $\beta$ -catenin.

### Statistical analysis

The experiments were repeated three times for each group. SPSS 17.0 statistical software was used for subsequent analysis. The analytical results were expressed using mean  $\pm$  SD, and a *t*-test was used when comparing paired groups. The difference was deemed statistically significant when *P* < 0.05.

## RESULTS

### Determination of biochemical indices of blood and liver

HA is a type of proteoglycan distributed around hepatic cells, and its content significantly increases in blood when hepatic fibrosis occurs<sup>[13]</sup>. Hyp in liver is an important part of the collagen fibers in liver, and the amount thereof also increases when hepatic fibrosis occurs<sup>[14]</sup>. Therefore, these two factors are important diagnostic indices of hepatic fibrosis. As shown in Table 2, the HA and Hyp of the CCl<sub>4</sub> group were significantly higher than those of the C group, which indicated, to some extent, that the hepatic fibrosis model had been successfully established. The HA and Hyp levels of the V, P, and V + P groups were much lower than those of the CCl<sub>4</sub> group. Among these groups, the V + P group showed the most significant reduction in HA and Hyp levels.

### Observation of the pathological sections of liver tissues

The Sirius-red-stained sections of each group were observed under the microscope. As illustrated in Figure 2A-F, mice in the CCl<sub>4</sub> group and the medication groups

**Table 2** Biochemical indices of blood and liver

| Groups           | HA (μg/L)                    | Hyp (μg/g)                  |
|------------------|------------------------------|-----------------------------|
| C                | 61 ± 20.6 <sup>bcd</sup>     | 195.6 ± 10.5 <sup>bcd</sup> |
| CCl <sub>4</sub> | 157.3 ± 44.3 <sup>acde</sup> | 503 ± 31.7 <sup>acde</sup>  |
| V                | 70.2 ± 12.9 <sup>b</sup>     | 375.2 ± 26.9 <sup>ab</sup>  |
| P                | 79.5 ± 11.6 <sup>b</sup>     | 361.3 ± 24.1 <sup>ab</sup>  |
| V + P            | 65 ± 12.1 <sup>b</sup>       | 353.7 ± 21.6 <sup>ab</sup>  |

In the comparison of the mean ± standard deviation (SD) of various groups, differences are statistically significant when  $P < 0.05$ , with  $n = 10$ . In addition, a, b, c, d, and e represent statistical differences vs C, CCl<sub>4</sub>, V, P, and V + P groups, respectively. C: Control; V: Vitamin; P: Puerarin.

showed larger areas of fibrosis in their liver tissues than in the C group. In addition, the areas of fibrosis of liver tissues in rats in each of the medicated groups were smaller than that of the CCl<sub>4</sub> group ( $P < 0.05$ ). Among the three groups, the V + P group presented the smallest area of fibrosis.

#### **mRNA levels of collagen I, collagen III, Wnt1, and β-catenin**

RT-PCR detection was conducted to test the mRNA levels of Collagen I, Collagen III, Wnt1, and β-catenin of the rats in each group. It can be seen from Table 3 that the mRNA levels of Collagen I, Collagen III, Wnt1, and β-catenin of liver tissues of rats in the CCl<sub>4</sub> group and the medicated groups are all higher than those of the C group. The mRNA levels of these indices of rats in the three medicated groups were all lower than those of the CCl<sub>4</sub> group ( $P < 0.05$ ). Among the medicated groups, the V + P group showed the lowest mRNA levels.

#### **Protein levels of Wnt1 and β-catenin of liver**

As the Wnt/β-catenin signalling pathway plays a significant role in the progression of hepatic fibrosis, a western blot assay was applied to detect the protein levels of Wnt1 and β-catenin in the liver. Figure 3 shows that the protein levels of Wnt1 and β-catenin in the liver tissues of rats in the CCl<sub>4</sub> group and the medicated groups were higher than the C group. The medicated groups showed lower protein levels than the CCl<sub>4</sub> group ( $P < 0.05$ ), among which the V + P group exhibited the lowest protein levels ( $P < 0.01$ ).

## **DISCUSSION**

Hepatic fibrosis is the inevitable pathological change during the development of chronic liver diseases. If it is not blocked, its further development would cause liver cirrhosis and even liver cancer. Hepatic fibrosis can be induced by many pathogenic factors, including viruses, parasites, alcohol, and some poisons such as CCl<sub>4</sub>. It is essentially a disturbance of the balance between the production and degradation of ECM outside hepatocytes. The ECM accumulates in the liver and its main component is collagen fibers<sup>[15,16]</sup>. HSCs, as the principal participants in the production and degradation

of ECM, when activated, are the key link necessary for the occurrence of hepatic fibrosis. Existing in the Disse space, HSCs mainly play their roles in metabolizing and storing vitamin A in normal conditions. They can synthesize and secrete small amounts of ECM and produce collagenase. When hepatic fibrosis occurs, HSCs in their resting state are activated. Active oxygen, lipid peroxide, and molecules secreted by nearby cells, such as activated Kupffer cells, and liver sinusoidal endothelial cells, as well as damaged hepatic cells, all can facilitate the activation of resting HSCs. After activation, HSCs have different morphologies, and the changes include the secretion of alpha smooth muscle actin (α-SMA), loss of vitamin A stored in cells, and increase in the rough endoplasmic reticulum. The activation of HSCs is also accompanied by a series of changes in genetic expression, including the appearance of receptors that can respond to paracrine stimulation on the cytomembrane of activated HSCs and a series of signalling cascade reactions in cells. The presence of the signalling cascade reactions in cells is beneficial to maintaining the phenotype of activated cells and controlling the occurrence of fibrosis, the proliferation of HSCs, and the increases in the transcription and translation of Collagen I and Collagen III<sup>[17-19]</sup>.

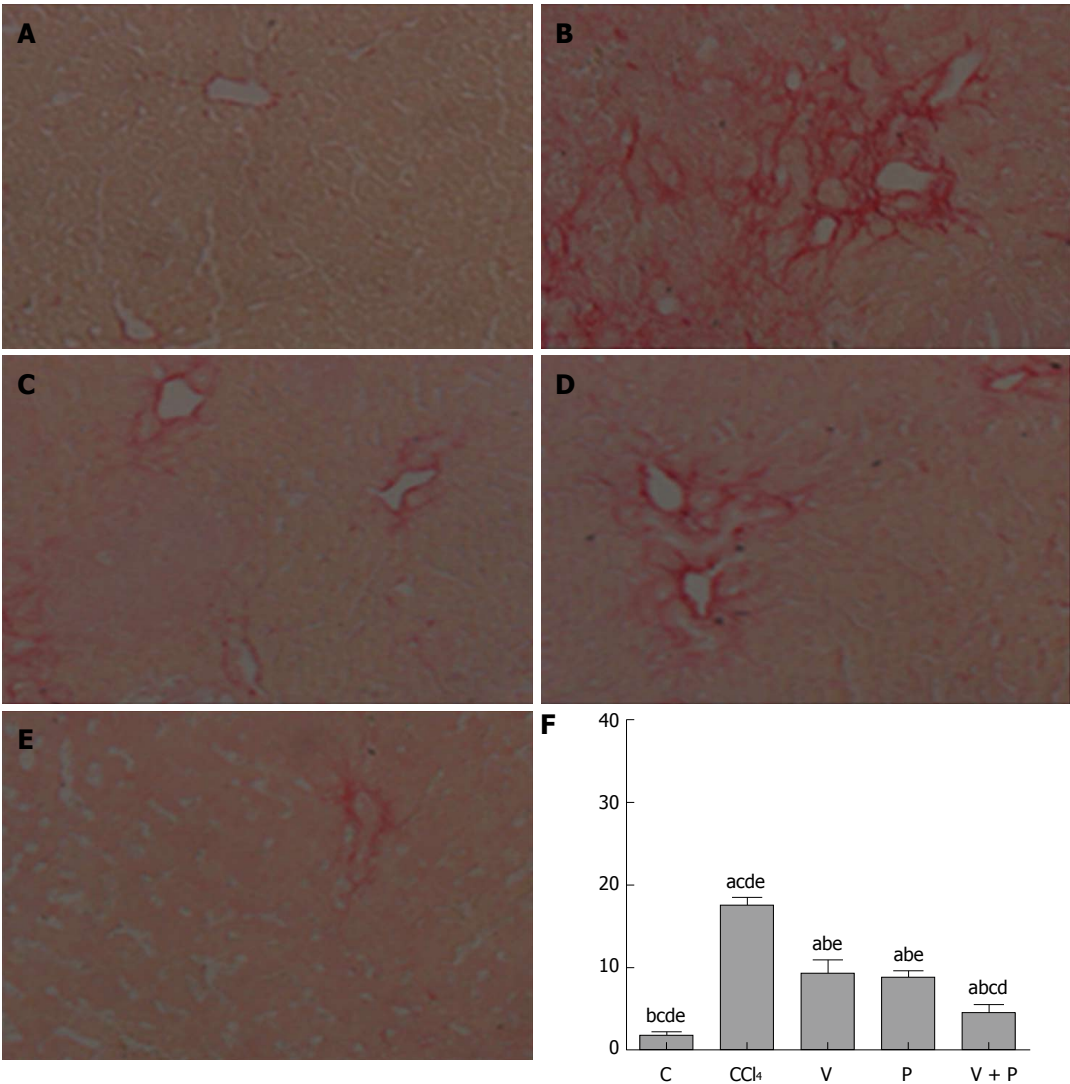
The canonical Wnt signal transduction pathway is also known as the Wnt/β-catenin signal transduction pathway. It is the pathway that has been the subject of most of the research to date. As a highly conserved signalling pathway activated during the evolution of some species, it is a basic pathway that is able to regulate cell proliferation and cell polarity, control cell fate, and maintain homeostasis in the embryo, and influence tissue development. Therefore, the pathway plays significant roles in various physiological processes including early development of animal embryos, organogenesis, and tissue regeneration, as shown in Figure 4<sup>[20]</sup>. When Wnt is deficient, β-catenin in the cytoplasm is generally degraded via Axin complexes; in addition, glycogen and casein kinase 1 (CK1) synthesize glycogen synthase kinase 3 (GSK3), which sequentially phosphorylates the amino terminus of β-catenin. As a result, β-catenin is recognized by β-Trcp and the sub-unit of E3 ubiquitin ligases and is then degraded by the ubiquitination pathway. The continuous removal of β-catenin inhibits the nuclear import of β-catenin and the combination of the protein family of T cell factor/lymphoid enhancer factor (TCF/LEF) with DNA, thus suppressing the transcription of Wnt target genes<sup>[21,22]</sup>. When Wnt ligands are bound with transmembrane Frizzled (Fz) receptors and their co-receptor-low-density lipoprotein receptor related protein 6 (LRP6) or similar LRP5, the Wnt/β-catenin signalling pathway and dishevelled Dsh/Dvl proteins in cells are activated. Consequently, the GSK-3β activity is suppressed, Axin falls off, and the formation of biodegradable composite film (mainly composed of Axin, APC, and GSK-3β) of β-catenin is inhibited. Therefore, β-catenin



**Table 3** The mRNA levels of collagen I, collagen III, Wnt1, and β-catenin of liver

| Groups           | Collagen I                  | Collagen III                | Wnt1                        | β-catenin                   |
|------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| C                | 1.17 ± 0.16 <sup>bcd</sup>  | 0.62 ± 0.15 <sup>bcd</sup>  | 0.56 ± 0.13 <sup>bcd</sup>  | 0.48 ± 0.21 <sup>bcd</sup>  |
| CCl <sub>4</sub> | 4.73 ± 0.76 <sup>acde</sup> | 2.47 ± 1.12 <sup>acde</sup> | 1.77 ± 0.32 <sup>acde</sup> | 2.16 ± 0.42 <sup>acde</sup> |
| V                | 1.97 ± 0.31 <sup>b</sup>    | 1.29 ± 0.19 <sup>ab</sup>   | 1.06 ± 0.35 <sup>ab</sup>   | 1.25 ± 0.33 <sup>ab</sup>   |
| P                | 1.75 ± 0.46 <sup>b</sup>    | 1.35 ± 0.17 <sup>ab</sup>   | 0.83 ± 0.19 <sup>ab</sup>   | 0.71 ± 0.46 <sup>ab</sup>   |
| V + P            | 1.53 ± 0.12 <sup>abcd</sup> | 1.03 ± 0.08 <sup>abcd</sup> | 0.61 ± 0.24 <sup>abcd</sup> | 0.59 ± 0.14 <sup>abcd</sup> |

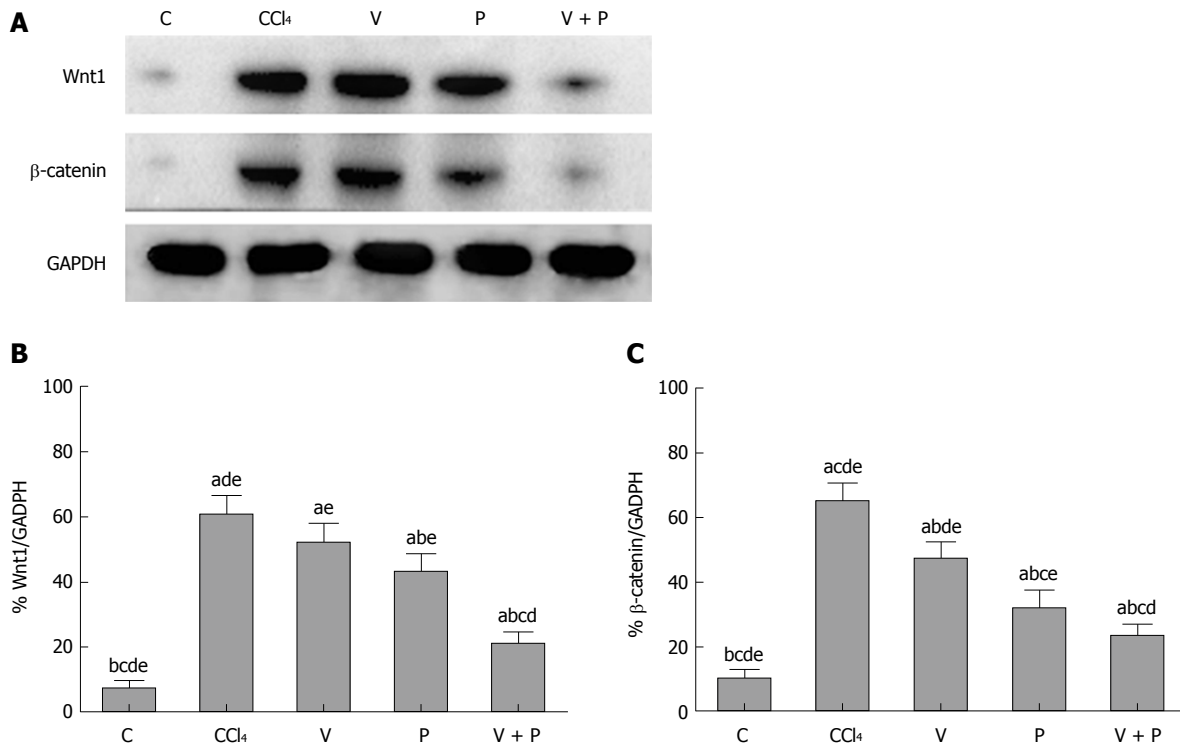
Differences are considered to exhibit statistical significance when  $P < 0.05$  ( $n = 10$ ) in the comparison of the mean ± standard deviation (SD) between various groups. a, b, c, d, and e indicate that there are statistical differences *vs* C, CCl<sub>4</sub>, V, P, and V + P groups, respectively. C: Control; V: Vitamin; P: Puerarin.



**Figure 2** Sirius red staining. A: Control (C) group; B: CCl<sub>4</sub> group; C: Vitamin D (V) group; D: Puerarin (P) group; E: V + P group (200 × magnification); F: Statistical graph of positive Sirius-red-stained areas (in the comparison of the mean ± SD of various groups, differences are statistically significant when  $P < 0.05$ ,  $n = 10$ . a, b, c, d, and e represent statistical differences *vs* C, CCl<sub>4</sub>, V, P, and V + P groups, respectively).

will aggregate, not be recognized, and degraded *via* the ubiquitination pathway<sup>[23,24]</sup>. As β-catenin accumulates to a certain level, it dissociates. Under these conditions, β-catenin is likely to undergo nuclear translocation, thus combining with the transcription factor TCF/LEF, forming transcriptional activation complex, and finally upregulating or downregulating the expressions of some downstream genes. At present, the known target

downstream genes include D1 (cyclin D1), c-myc, MMP7, survivin, CD44, and growth factor, and new target genes are constantly being discovered<sup>[25,26]</sup>. In the Wnt/β-catenin pathway, Wnt1 is the key gene participating in the aggregation and disappearance of β-catenin and is closely related to the occurrence and development of hepatic fibrosis and tumors. The Wnt/β-catenin pathway and the activation and



**Figure 3** The protein levels of Wnt1 and  $\beta$ -catenin of liver tissues in rats in the CCl<sub>4</sub> group and the medicated groups were higher than the Control (C) group. A: Detection of protein levels of Wnt1, and  $\beta$ -catenin using western blot assay; B: Grey analysis of Wnt1; C: Grey analysis of  $\beta$ -catenin [in the comparison of mean  $\pm$  standard deviation (SD) of various groups, differences are statistically significant when  $P < 0.05$ ,  $n = 10$ . In addition, a, b, c, d, and e represent statistical differences vs C, CCl<sub>4</sub>, V, P, and V + P groups, respectively]. C: Control; V: Vitamin; P: Puerarin.

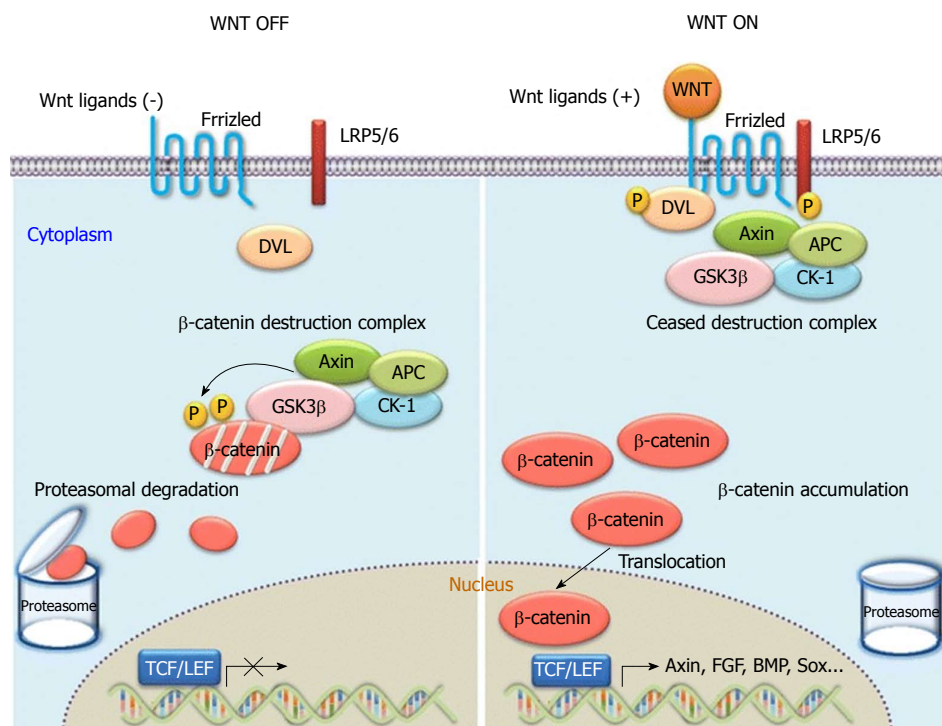
proliferation of HSCs are therefore associated with hepatic fibrosis. The Wnt/ $\beta$ -catenin pathway, dominated as it is by Wnt and  $\beta$ -catenin, plays a significant role in regulating the activation of HSCs and the secretion of Collagen I and III. Although the mechanism *via* which the Wnt/ $\beta$ -catenin signalling pathway takes part in hepatic fibrosis by activating HSCs is not completely understood, activated HSCs, as well as Wnt1 and  $\beta$ -catenin, have become important targets in anti-hepatic fibrosis therapy.

Most of the vitamin D in the body is produced by the liver, which is also one of the target organs of vitamin D. Previous studies have revealed that vitamin D is capable of ameliorating CCl<sub>4</sub>-induced hepatic fibrosis in mice. *In vitro* experiments have shown that this is probably because it reduces the activation of HSCs and the secretion of collagen fibers. Puerarin, as a type of flavonoid drug that is widely applied in clinical practice, is also able to alleviate CCl<sub>4</sub>-induced hepatic fibrosis (according to the evidence of existing research): however, there is no report on the issue as to whether the Wnt/ $\beta$ -catenin signalling pathway can be significantly suppressed after the combined use of vitamin D and puerarin. Whether using vitamin D or puerarin alone, their protective effects on the liver during the progression of hepatic fibrosis have been found to be closely associated with their functions of suppressing HSCs. Our preliminary research revealed that the combined use of the two drugs is able to impart

greater protective function, so we speculated that the mechanism of the combined use is probably related to HSCs, Wnt, and  $\beta$ -catenin.

The research demonstrated that CCl<sub>4</sub> greatly increased the HA level in blood and Hyp level in rat liver. By observing the Sirius-red-stained specimens under a microscope, it was found that CCl<sub>4</sub> caused the deposition of collagen fibers in the liver of the rats. All these results indicated that the rat model of hepatic fibrosis was successfully established. Vitamin D and puerarin can both reduce the HA level in blood and Hyp level in liver of the rats, thus greatly decreasing the amount of collagen fibers in the liver. The combined use of vitamin D and puerarin most significantly reduced various damage indices and alleviated the deposition of collagen fibers, which proved that the two drugs have synergistic effects. Meanwhile, we detected the mRNA and protein levels of Collagen I, Collagen III, Wnt1, and  $\beta$ -catenin molecules in the liver of the rats. The results indicated that both vitamin D and puerarin are capable of decreasing the mRNA and protein levels of these molecules. Likewise, the most significant reduction effect was also observed in the V + P group. This suggested that the protective effect of the combined use of vitamin D and puerarin in the progression of hepatic fibrosis is closely associated with the functions of silencing the Wnt1/ $\beta$ -catenin pathway, suppressing the activation of HSCs, and decreasing the secretion of collagen fibers.

The research investigated the protective effect of the



**Figure 4 Overview of WNT/β-catenin signalling.** Without WNT signalling ("WNT OFF"), the destruction complex phosphorylates cytosolic β-catenin, and phosphorylated β-catenin is recognized and degraded by the proteasomes. With active WNT signalling ("WNT ON"), the function of the "destruction complex" is inhibited to phosphorylate cytosolic β-catenin. Then unphosphorylated β-catenin accumulates in the cytosol, translocates into the nucleus, and activates WNT target gene expression, such as the targets of the T-cell factor and lymphoid enhancer factor-1 (TCF/LEF1) family of transcription factors.

combined use of vitamin D and puerarin against CCl<sub>4</sub>-induced hepatic fibrosis in rats. The results revealed that the combined use of the two drugs exhibited a superior anti-hepatic fibrosis effect compared to that of each of the agents used as a single medication. It was preliminarily found that the protective mechanism of the combined use was related to the Wnt/β-catenin pathway, which provided a useful reference for those in clinical practice. However, the regulatory mechanism is complex and involves multiple factors and pathways. Other mechanisms of action warrant further investigation.

## ARTICLE HIGHLIGHTS

### Research background

Hepatic fibrosis is seriously endangering the safety of life. At present, there is no ideal way to treat hepatic fibrosis. The combination of vitamin D and puerarin can improve the effects of anti-hepatic fibrosis, but the mechanism is not clear. Therefore, the aim of this study is to explore the mechanism of the combined use of vitamin D and puerarin in the treatment of hepatic fibrosis, so as to improve the theoretical basis for the treatment of hepatic fibrosis.

### Research motivation

The combination of vitamin D and puerarin can improve the effect of anti-hepatic fibrosis, but its mechanism is not clear. The Wnt/β-catenin pathway is closely related to liver fibrosis. In this study, we found that vitamin D and puerarin are closely related to the regulation of Wnt/β-catenin pathway in hepatic fibrosis, which provided a useful reference for those in clinical practice.

### Research objectives

The research aimed to reveal the protective mechanism of the combined use of vitamin D and puerarin in the progression of hepatic fibrosis induced by carbon

tetrachloride (CCl<sub>4</sub>).

### Research methods

In this study, a Wistar rat model of hepatic fibrosis was constructed by CCl<sub>4</sub>. Vitamin D combined with puerarin was used in the treatment of hepatic fibrosis rats, and the liver pathology and the genes related to the Wnt/β-catenin pathway were detected. The protective mechanism of the combined use of vitamin D and puerarin in the progression of hepatic fibrosis was explored at the molecular level.

### Research results

The research demonstrated that CCl<sub>4</sub> greatly increased the HA level in blood and Hyp level in the rat liver. CCl<sub>4</sub> caused the deposition of collagen fibers in the liver of the rats. Vitamin D and puerarin can both reduce the HA level in blood and Hyp level in liver of the rats, thus greatly decreasing the amount of collagen fibers in the liver. The combined use of vitamin D and puerarin most significantly reduced the various damage indices, and alleviated the deposition of collagen fibers, which proved that the two drugs had synergistic effects.

### Research conclusions

The combined application of vitamin D and puerarin is capable of alleviating the CCl<sub>4</sub>-induced hepatic fibrosis of rats.

### Research perspectives

We have learned to induce an animal model of hepatic fibrosis and study the drug effects by the animal model, but also because we were not skilled enough in the experimental technique and spent more time doing repetitive work. The future research direction is to explore the prevention and treatment measures of hepatic fibrosis. The drug targets will be studied by transcriptome, proteomics and other methods.

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## Retrospective Study

# Frequency, types, and treatment of anemia in Turkish patients with inflammatory bowel disease

Göksel Bengi, Hatice Keyvan, Seda Bayrak Durmaz, Hale Akpınar

Göksel Bengi, Hale Akpınar, Division of Gastroenterology, Department of Internal Medicine, Faculty of Medicine, Dokuz Eylül University, İzmir 35360, Turkey

Hatice Keyvan, Seda Bayrak Durmaz, Department of Internal Medicine, Faculty of Medicine, Dokuz Eylül University, İzmir 35360, Turkey

ORCID number: Göksel Bengi (0000-0002-7859-4094); Hatice Keyvan (0000-0002-7032-6124); Seda Bayrak Durmaz (0000-0001-8272-5686); Hale Akpınar (0000-0002-8734-2518).

**Author contributions:** Bengi G, Keyvan H, Durmaz SB, and Akpınar H contributed equally to this work, designed the research, drafted the manuscript, and provided administrative and technical support.

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**Informed consent statement:** Informed consent was provided by all participants.

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**Correspondence to:** Göksel Bengi, MD, PhD, Assistant Professor, Department of Gastroenterology, Dokuz Eylül University Hospital, İnciraltı, İzmir 35000, Turkey. [goksel.bengi@deu.edu.tr](mailto:goksel.bengi@deu.edu.tr)  
Telephone: +90-532-4626972  
Fax: +90-232-4123799

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## Abstract

### AIM

To specify the type and prevalence of anemia along with a treatment approach for inflammatory bowel disease (IBD).

### METHODS

We conducted a retrospective study on 465 patients who were diagnosed with IBD and followed up at our hospital from June 2015 to June 2016 [male: 254, female: 211; average age:  $47 \pm 14.4$ ; Crohn's disease (CD): 257, Ulcerative Colitis (UC): 208]. Epidemiological and clinical data, such as sex, age, age of diagnosis, type of IBD, disease extension, disease behavior and duration, treatments for IBD and anemia, and surgical history were obtained for each patient. Per World Health Organization guidelines, anemia was diagnosed for males if hemoglobin values were less than 13 g/dL and for females if hemoglobin values were less than 12 g/dL.

### RESULTS

We determined that 51.6% of the patients had anemia, which was more frequent in women than men (64% vs 41.3%,  $P < 0.001$ ). Anemia frequency was higher in CD cases (57.6%) than in UC cases (44.2%) ( $P = 0.004$ ). CD involvements were as follows: 48.2% in ileal involvement, 19% in colonic involvement, and 32.8% in ileocolonic involvement. Furthermore, 27.5% of UC patients had proctitis (E1) involvement, 41% of

them had involvement in left colitis (E2), and 31.5% had pancolitis involvement. There was no significant relationship between anemia frequency and duration of disease ( $P = 0.55$ ). Iron deficiency anemia (IDA) was the most common type of anemia in this cohort. Moreover, because anemia parameters have not been evaluated during follow-up of 15.3% of patients, the etiology of anemia has not been clarified. Fifty percent of patients with anemia received treatment. Twenty-three percent of IDA patients had oral iron intake and forty-one percent of IDA patients had parenteral iron treatment. Fifty-three percent of patients who were suffering from megaloblastic anemia received B<sub>12</sub>/folic acid treatment.

### CONCLUSION

We found out that almost half of all IBD patients (51.6%) had anemia, the most frequent of which was IDA. Almost half of these patients received treatment. We should increase the treatment rate in our IBD patients that have anemia.

**Key words:** Anemia; Inflammatory bowel disease; Anemia of iron deficiency

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**Core tip:** We conducted a retrospective study on 465 patients, who were diagnosed with inflammatory bowel disease (IBD). We determined that 51.6% of patients had anemia, which was more frequent in women than men. Anemia frequency was higher in Crohn's disease cases than in ulcerative colitis cases. No relation has been found between the presence of anemia and disease duration. Iron deficiency anemia was the most common type of anemia. The factors that are related to anemia among IBD patients are being female, drug therapy (corticosteroids, AZA/MTX, Anti-TNF), and high C-reactive protein levels. Fifty percent of patients with anemia received treatment.

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## INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic idiopathic disease with a relapsing and remitting course. There are two major types of IBD, including Crohn's disease (CD) and ulcerative colitis (UC). The most common extraintestinal finding seen in IBD patients is anemia, which decreases both the quality of life and the ability to work. Fatigue and weakness are the most common

complaints reported in IBD-related anemia. Moreover, IBD-related anemia has been associated with frequent hospitalizations, late hospital discharge, increased health expenditures, co-morbidity for other diseases [e.g., transfusion-related Hepatitis C virus (HCV), etc.], and most importantly, a significant increase in the risk of mortality<sup>[1]</sup>.

The prevalence of anemia is higher in IBD patients than in the general population, and ranges between 6% and 74%<sup>[2,3]</sup>. According to a review of 22 articles<sup>[2]</sup>, the mean prevalence of anemia among IBD patients is 17% (95%CI: 16-18). In a meta-analysis<sup>[4]</sup>, the prevalence of anemia in 2192 IBD patients was reported as 24% (27% in CD patients and 21% in UC patients). A recent study by Koutroubakis *et al.*<sup>[5]</sup> including 1821 patients (1077 CD, 744 UC) reported the prevalence of anemia as 50.1% (CD: 53.3%, UC: 44.7%). The first study to report on the incidence of anemia in Turkish patients with IBD reported that 58.2% of 941 patients (62.1% of 375 CD patients and 55.7% of 566 UC patients) had anemia at least once during an 18-year follow-up period<sup>[6]</sup>.

The most common causes of anemia in IBD are iron deficiency anemia (IDA) and chronic disease anemia (CDA)<sup>[6-10]</sup>. Other causes of anemia in IBD include macrocytic anemias (such as vitamin B<sub>12</sub> deficiency and/or folate deficiency), hemolytic anemia, and drug-related bone marrow suppression.

Although IBD-related anemia has a relatively high prevalence, its diagnosis and treatment is generally overlooked. Iron therapy is recommended for all patients with IDA-related IBD, and the treatment should aim to return the patient to normal hemoglobin levels and provide adequate iron storage<sup>[11]</sup>. Recently, intravenous iron therapy has been recommended for the treatment of IDA-related IBD<sup>[12]</sup>. This is because intravenous iron treatment exerts its effects quickly, particularly among those who have active disease, have been intolerant to previous oral iron therapy, have severe anemia (Hb < 10 g/dL), and require erythropoiesis-stimulating agents<sup>[13]</sup>.

The current study aimed to determine the frequency and types of anemia in IBD patients, to determine the relationship between anemia and disease characteristics, and to determine the most effective treatment approach.

## MATERIALS AND METHODS

### Study design and data collection

**Patients:** This study retrospectively evaluated 465 patients who were diagnosed with IBD and followed-up between June 2015 and June 2016 in the Gastroenterology/IBD outpatient clinic or ward of Dokuz Eylül University, Medical Faculty Hospital.

The IBD diagnoses were made in accordance with the new European Crohn's and Colitis Organization (ECCO) guidelines, and were confirmed according to standard clinical, endoscopic, histologic, and radiological criteria<sup>[14,15]</sup>. In order to obtain epidemiological and

**Table 1** Types of anemia according to iron parameters

|                          | Ferritin (ng/mL) | Transferrin saturation (%) | CRP (5 mg/L) |
|--------------------------|------------------|----------------------------|--------------|
| IDA                      | < 30             | < 20                       | -            |
| CDA                      | > 100            | < 20                       | > 5          |
| Mixed anemia (IDA + CDA) | > 30 and < 100   | < 20                       |              |

IDA: Iron deficiency anemia; CDA: Chronic disease anemia; CRP: C reactive protein.

clinical data, the following data were recorded for each patient: sex, age, age at diagnosis, type of IBD, disease extension, disease behavior and duration, treatments for IBD and anemia, and surgical history. Patients were excluded from this study if they had indeterminate colitis, were pregnant, were monitored for less than one year, or had diseases such as chronic renal insufficiency, gastrectomy, hematological diseases, *etc.* Demographic and clinical data as well as endoscopic activities were obtained from hospital records.

**Definition of anemia:** We used the World Health Organization guidelines to diagnose anemia in our IBD patients. Males were diagnosed with anemia if they had hemoglobin values less than 13 g/dL, and females were diagnosed if they had hemoglobin values less than 12 g/dL<sup>[16]</sup>. Severe anemia was defined as having Hb values below 10 g/dL for both sexes. We evaluated the lowest hemoglobin levels of each patient during follow-up, as well as iron levels and other anemia parameters. The following were obtained from each patient's laboratory records: hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), serum ferritin, serum iron level, transferrin saturation (TS), serum iron binding capacity (SIBC), folic acid, vitamin B<sub>12</sub>, CRP, and albumin.

Three main classifications of anemia were selected in accordance with the European consensus on anemia in IBD, including IDA, CDA, and mixed anemia<sup>[17]</sup> (Table 1). Aside from these, other causes of anemia were determined by examining the peripheral smear, and in cases with suspected macrocytic anemia, vitamin B<sub>12</sub> and folic acid levels were evaluated. Additionally, medications that may cause macrocytic anemia (*i.e.*, thiopurines and sulfasalazine) were also taken into account. In cases with suspected hemolytic anemia, reticulocyte ratio and haptoglobin levels were evaluated.

### Statistical analysis

In our current study, variables indicated by census are presented as percentage distributions, and variables indicated by measurements are presented as means and standard deviations. In univariate analyses, Chi Square and Fisher's exact test were used to compare the variables indicated by census. The variables indicated by measurement were compared by Student's *t*-test. The logistic regression model was used in multivariate analysis. Values of *P* < 0.05 were considered significant.

All analyses were made using the Statistical package for the Social Sciences (SPSS) (version 22.0; SPSS Inc., Chicago, IL, United States).

### Ethical considerations

This study was approved by Dokuz Eylül University School of Medicine's non-invasive clinical research ethics committee (15.06.2017 3387-GOA). Patient information was kept confidential, and the study was conducted according to the Helsinki declaration.

## RESULTS

### Patient characteristics

This study included the data from 465 IBD patients [254 male (54.6%) and 211 female (45.4%)] who were newly diagnosed or were being followed-up with in our hospital. Of these patients, 55.3% were diagnosed with CD and 44.7% with UC. The mean age at IBD diagnosis was 40.2 ± 13.9 years (39.8 ± 13.7 years in CD patients, 40.7 ± 14.6 years in UC patients, *P* = 0.46). The patient characteristics are presented in Table 2. There was no significant difference between mean disease duration of CD (6.45 years) and UC (7.36 years) (*P* = 0.07).

Among patients with CD, 48.2% had ileal involvement (L1), 19% had colonic involvement (L2), and 32.8% had ileocolonic involvement (L3). Isolated upper GI involvement (L4) was not observed in any of the patients. In terms of CD behavior, 60.9% of patients had inflammatory type CD (B1), 16.5% had structuring type CD (B2), and 22.6% had penetrating type CD (B3).

In patients with UC, 27.5% had proctitis involvement (E1), 41% had left colitis involvement (E2), and 31.5% had pancolitis involvement (Table 2).

### Frequency and type of anemia among IBD patients

In our current study, 51.6% (*n* = 240) of the patients had anemia. Anemia frequency was higher in CD (57.6%) than in UC (44.2%) (*P* = 0.004). The mean hemoglobin concentration was 12.3 g/dL in IBD patients, and was significantly lower in those with CD (12.1 g/dL) than in those with UC (12.5 g/dL) (*P* = 0.03). The frequency of anemia and hematological profiles at the time of IBD diagnosis is shown in Table 3.

Anemia was more common among women than men (64% vs 41.3%, *P* < 0.001) (Table 4). Severe anemia (Hb < 10 g/dL) was observed in 21.6% of patients with CD and 9.8% of patients with UC (*P*

**Table 2** Demographic data of inflammatory bowel disease patients *n* (%)

|                           | IBD overall           | Crohn's disease       | Ulcerative colitis    | <i>P</i> value |
|---------------------------|-----------------------|-----------------------|-----------------------|----------------|
| <i>n</i>                  | 465                   | 257 (55.3)            | 208 (44.7)            |                |
| Age (yr) mean (SD; range) | 47.1 (14.3; 18-83)    | 46.2 (13.4; 17-78)    | 48 (15.3; 12-83)      | 0.183          |
| Sex (male/female)         | 254 (54.6)/211 (45.4) | 153 (59.5)/104 (40.5) | 101 (48.6)/107 (51.4) | 0.019          |
| Disease characteristics   |                       |                       |                       |                |
| Age at diagnosis (yr)     | 40.2                  | 39.7                  | 40.7                  | 0.46           |
| Location of the disease   |                       |                       |                       |                |
| L1                        |                       | 122 (48.2)            |                       |                |
| L2                        |                       | 48 (19)               |                       |                |
| L3                        |                       | 83 (32.8)             |                       |                |
| L4                        |                       | -                     |                       |                |
| Disease behavior          |                       |                       |                       |                |
| B1                        |                       | 148 (60.9)            |                       |                |
| B2                        |                       | 40 (16.5)             |                       |                |
| B3                        |                       | 55 (22.6)             |                       |                |
| Disease extension         |                       |                       |                       |                |
| E1                        |                       |                       | 49 (27.5)             |                |
| E2                        |                       |                       | 73 (41)               |                |
| E3                        |                       |                       | 56 (31.5)             |                |
| Drugs                     |                       |                       |                       |                |
| SZP/5-ASA                 | 360 (77.4)            | 171 (66.5)            | 189 (90.6)            | < 0.001        |
| Corticosteroids           | 55 (11.8)             | 30 (11.7)             | 25 (12)               | 0.90           |
| Budesonide                | 17 (3.7)              | 15 (5.8)              | 2 (1)                 | 0.005          |
| AZA / MTX                 | 200 (43)              | 155 (60.3)            | 45 (21.6)             | < 0.001        |
| IFX/ADA                   | 81 (17.4)             | 72 (28)               | 9 (4.3)               | < 0.001        |
| Antibiotic                | 92 (19.8)             | 68 (26.5)             | 24 (11.5)             | < 0.001        |
| Surgery                   | 70 (15.1)             | 60 (23.3)             | 10 (4.8)              | < 0.001        |

IBD: Inflammatory bowel disease; L: Disease location (for Crohn's disease); L1: Ileal disease; L2: Colonic disease; L3: Ileocolonic disease; L4: Upper gastrointestinal tract disease; B: Disease behavior (for Crohn's disease); B1: Inflammatory disease; B2: Stricturing disease; B3: Penetrating disease; E: Disease extension (for ulcerative colitis); E1: Ulcerative proctitis; E2: Left-sided ulcerative colitis; E3: Extensive disease; SZP: Sulphasalazine; 5-ASA: 5-aminosalicylate; AZA: Azathiopurine; MTX: Methotrexate; IFX: Infliximab; ADA: Adalimumab.

**Table 3** Prevalence of anemia and hematological profile at the time of diagnosis in patients with inflammatory bowel disease *n* (%)

|                                    | IBD overall          | Crohn's disease      | Ulcerative colitis   | <i>P</i> value |
|------------------------------------|----------------------|----------------------|----------------------|----------------|
| Anemia                             | 240 (51.6)           | 148 (57.6)           | 92 (44.2)            | 0.005          |
| Severe anemia                      | 64 (16.4)            | 47 (21.6)            | 17 (9.8)             | 0.002          |
| Hemoglobin (g/dL) mean (SD; range) | 12.3 (2.1; 5.3-17.9) | 12.1 (2.2; 5.3-16.8) | 12.5 (1.9; 7.1-17.9) | 0.03           |
| Hematocrit (%) mean (SD; range)    | 37.41 (6.1; 10-53)   | 36.8 (6.6; 10-52)    | 38.16 (5.3; 22-53)   | 0.02           |
| MCV (fL) mean (SD; range)          | 84.54 (7.7; 57-112)  | 84.75 (8.3; 58-112)  | 84.28 (7; 57-100)    | 0.46           |
| Iron (µg/dL) mean (SD; range)      | 43.76 (30.3; 4-160)  | 43.26 (30.4; 4-126)  | 44.5 (30; 4-160)     | 0.73           |
| Ferritin (µg/dL) mean (SD; range)  | 40.3 (74; 2-754)     | 48.96 (91; 2-754)    | 27.26 (37; 2-167)    | 0.01           |
| TS (%) mean (SD; range)            | 15.35 (13; 2-100)    | 15.14 (12.7; 2-100)  | 15.67 (12; 2-92)     | 0.9            |
| CRP (mg/L) mean (SD; range)        | 13 (29; 1-289)       | 17.1 (32.8; 0-289)   | 7.9 (7.93; 1-242)    | 0.001          |
| Albumin mean (SD; range)           | 4.2 (0.4; 1.9-5.0)   | 4.1 (0.4; 1.9-4.9)   | 4.1 (0.4; 2.0-5.0)   | 0.17           |

MCV: Mean corpuscular volume; TS: Transferrin saturation; CRP: C reactive protein.

= 0.002). No relationship was found between the presence of anemia and disease duration ( $P = 0.55$ ).

IDA was the most common type of anemia (29.9%). The frequencies of CDA and mixed anemia (IDA + CDA) were 8% and 3.4%, respectively. In addition, vitamin B<sub>12</sub>/folic acid deficiency anemia was observed in 14% of the patients (Figure 1). Moreover, since anemia parameters were not evaluated during the follow-up of 15.3% of our IBD patients, the etiology of anemia was not clarified in these patients.

#### Factors related to anemia among IBD patients

Results of the current study indicate that anemia in IBD

patients is more common among women than men, regardless of disease type or age. While elevated CRP levels (> 5 mg/L) are indicative of active disease, CD and UC patients with CRP levels > 5 mg/L had significantly higher rates of anemia compared to those with lower levels of CRP (70.4% vs 45.8% for CD patients,  $P < 0.001$ , and 54.7% vs 36.8% for UC patients,  $P = 0.006$ ). The factors related to anemia in IBD are summarized in Table 5. Data from this study indicate that disease type and duration do not have a significant effect on the frequency of anemia in CD.

Corticosteroid users had a higher frequency of anemia than non-users (80% vs 47.8%,  $P < 0.001$ ).



**Table 4** Evaluation of disease characteristics according to types of anemia in inflammatory bowel disease *n* (%)

|                       | Anemia     | IDA        | CDA       | Mixed anemia | B <sub>12</sub> /Folic acid anemia | Other anemia |
|-----------------------|------------|------------|-----------|--------------|------------------------------------|--------------|
| Sex                   |            |            |           |              |                                    |              |
| M                     | 105 (41.3) | 48 (18.9)  | 18 (7.1)  | 8 (3.8)      | 34 (13.4)                          | 35 (13.8)    |
| F                     | 135 (64)   | 91 (43.1)  | 19 (9)    | 8 (3.1)      | 31 (14.6)                          | 36 (17.1)    |
| <i>P</i> value        | < 0.001    | < 0.001    | 0.44      | 0.7          | 0.6                                | 0.32         |
| Disease duration (yr) |            |            |           |              |                                    |              |
| A+                    | 6.6        | 6.7        | 6.1       | 5.9          | 5.9                                | 6.8          |
| A-                    | 7.1        | 6.9        | 6.9       | 6.9          | 7                                  | 6.9          |
| <i>P</i> value        | 0.55       | 0.45       | 0.31      |              | 0.14                               | 0.36         |
| Disease type          |            |            |           |              |                                    |              |
| CD                    | 148 (57.6) | 84 (32.7)  | 31 (12.1) | 13 (5.1)     | 50 (19.4)                          | 39 (15.2)    |
| UC                    | 92 (44.2)  | 55 (26.4)  | 6 (2.9)   | 3 (1.4)      | 15 (7.2)                           | 32 (15.4)    |
| <i>P</i> value        | 0.004      | 0.14       | < 0.001   | 0.03         | 0.001                              | 0.95         |
| Disease localization  |            |            |           |              |                                    |              |
| L1                    | 66 (54.1)  | 34 (27.9)  | 13 (10.7) | 5 (4.1)      | 25 (20.5)                          | 19 (15.6)    |
| L2                    | 29 (60.4)  | 17 (35.4)  | 8 (16.7)  | 1 (2.1)      | 3 (6.3)                            | 9 (18.8)     |
| L3                    | 50 (60.2)  | 31 (37.3)  | 9 (13.8)  | 6 (7.2)      | 22 (26.5)                          | 10 (12)      |
| <i>P</i> value        | 0.6        | 0.32       | 0.51      |              | 0.02                               | 0.56         |
| Disease behavior      |            |            |           |              |                                    |              |
| B1                    | 90 (60.8)  | 53 (35.8)  | 18 (12.2) | 8 (5.4)      | 23 (15.6)                          | 25 (16.9)    |
| B2                    | 27 (67.5)  | 12 (30)    | 5 (12.5)  | 1 (2.5)      | 14 (35)                            | 6 (15)       |
| B3                    | 26 (47.3)  | 16 (29.1)  | 8 (14.5)  | 4 (7.3)      | 12 (21.8)                          | 6 (10.9)     |
| <i>P</i> value        | 0.1        | 0.59       | 0.9       |              | 0.02                               | 0.57         |
| Disease extension     |            |            |           |              |                                    |              |
| E1                    | 16 (32.7)  | 6 (12.2)   | 1 (2)     | 0            | 2 (4.1)                            | 9 (18.4)     |
| E2                    | 32 (48.8)  | 19 (26)    | 2 (2.7)   | 2 (2.7)      | 6 (8.2)                            | 12 (16.4)    |
| E3                    | 35 (62.5)  | 26 (46.4)  | 3 (5.4)   | 1 (1.8)      | 6 (10.7)                           | 6 (10.7)     |
| <i>P</i> value        | 0.008      | < 0.001    |           |              |                                    | 0.51         |
| Corticosteroid use    |            |            |           |              |                                    |              |
| +                     | 44 (80)    | 28 (50.9)  | 10 (18.2) | 4 (7.3)      | 15 (27.3)                          | 10 (18.2)    |
| None                  | 196 (47.8) | 111 (27.1) | 27 (6.6)  | 12 (2.9)     | 50 (12.2)                          | 61 (14.9)    |
| <i>P</i> value        | < 0.001    | < 0.001    | 0.003     | 0.097        | 0.003                              | 0.52         |
| AZA/MTX use           |            |            |           |              |                                    |              |
| +                     | 129 (64.5) | 73 (36.5)  | 25 (12.5) | 11 (5.5)     | 45 (22.5)                          | 36 (18)      |
| None                  | 111 (41.9) | 66 (24.5)  | 12 (4.5)  | 5 (1.9)      | 20 (7.5)                           | 35 (13.2)    |
| <i>P</i> value        | < 0.001    | 0.007      | 0.002     | 0.03         | < 0.001                            | 0.15         |
| Anti-TNF use          |            |            |           |              |                                    |              |
| +                     | 56 (69.1)  | 38 (46.9)  | 13 (16)   | 7 (8.6)      | 21 (26)                            | 11 (13.6)    |
| None                  | 184 (47.9) | 101 (26.3) | 24 (6.3)  | 9 (2.3)      | 44 (11.5)                          | 60 (15.6)    |
| <i>P</i> value        | 0.001      | < 0.001    | 0.003     | 0.01         | 0.002                              | 0.64         |
| Surgery               |            |            |           |              |                                    |              |
| +                     | 44 (62.9)  | 23 (32.9)  | 11 (15.7) | 5 (7.19)     | 15 (21.4)                          | 14 (20)      |
| None                  | 196 (49.6) | 116 (29.4) | 26 (6.6)  | 11 (2.8)     | 50 (12.6)                          | 57 (14.4)    |
| <i>P</i> value        | 0.041      | 0.55       | 0.009     | 0.07         | 0.55                               | 0.23         |
| CRP (mg/L)            |            |            |           |              |                                    |              |
| < 5                   | 109 (41.3) | 70 (26.5)  | 7 (2.7)   | 3 (1.1)      | 31 (11.8)                          | 29 (11)      |
| > 5                   | 120 (65.6) | 63 (34.4)  | 29 (15.8) | 12 (6.6)     | 30 (16.4)                          | 39 (21.3)    |
| <i>P</i> value        | 0.001      | 0.07       | 0.001     | 0.002        | 0.37                               | 0.003        |

M: Male; F: Female; A+: Anemia present; A-: Anemia absent; IBD: Inflammatory bowel disease; L: Disease location (for Crohn's disease); L1: Ileal disease; L2: Colonic disease; L3: Ileocolonic disease; L4: Upper gastrointestinal tract disease; B: Disease behavior (for Crohn's disease); B1: Inflammatory disease; B2: Stricturing disease; B3: Penetrating disease; E: Disease extension (for ulcerative colitis); E1: Ulcerative proctitis; E2: Left-sided ulcerative colitis; E3: Extensive disease; SZP: Sulphasalazine; 5-ASA: 5-aminosalicylate; AZA: Azathiopurine; MTX: Methotrexate; IFX: Infliximab; ADA: Adalimumab.

Further, those using immunomodulator therapy (such as AZA and MTX) also had higher rates of anemia than non-users (64.5% vs 41.9%,  $P < 0.001$ ). Vitamin B<sub>12</sub>/folic acid deficiency anemia was the most common type of anemia in this immunomodulator users group. On the other hand, anemia rates were significantly higher among patients who did not use anti-TNF, compared to those who did use anti-TNF (69.1% vs 30.9%,  $P = 0.001$ ). Further, those who had undergone previous surgeries had higher rates of anemia than those who did not (62.9 % vs 37.1%,  $P = 0.04$ ).

In UC patients, left colon involvement and pancolitis involvement were associated with a higher incidence of anemia than proctitis involvement. Also in the UC patients, as disease duration increased, the incidence of anemia significantly increased ( $P = 0.008$ ) (Table 4). IDA is significantly more prevalent in UC with pancolitis involvement compared to other types of disease involvement ( $P < 0.001$ ).

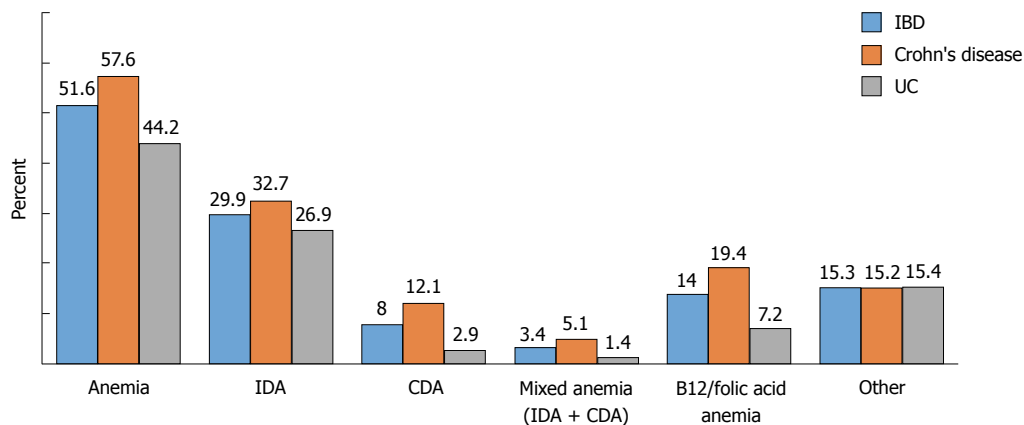
#### Anemia management in patients with IBD

Approximately 50.4% of all of the IBD patients who

**Table 5** Factors associated with anemia by logistic regression in patients with Crohn's disease and ulcerative colitis at the diagnosis

|                    | Odds ratio | 95%CI      | P value |
|--------------------|------------|------------|---------|
| All IBD patients   |            |            |         |
| Sex (female)       | 3.19       | 2.07-4.91  | < 0.001 |
| Corticosteroids    | 3.21       | 1.52-6.80  | 0.002   |
| AZA/MTX            | 2.28       | 1.48-3.49  | < 0.001 |
| Anti-TNF (INF/ADA) | 2.32       | 1.30-4.11  | 0.004   |
| Elevated CRP       | 2.75       | 1.78-4.24  | < 0.001 |
| Crohn's disease    |            |            |         |
| Sex (female)       | 4.10       | 2.19-7.68  | < 0.001 |
| Corticosteroids    | 4.06       | 1.27-12.96 | 0.018   |
| AZA/MTX            | 2.30       | 1.26-4.20  | 0.006   |
| Anti-TNF (INF/ADA) | 2.34       | 1.20-4.53  | 0.012   |
| Elevated CRP       | 3.05       | 1.67-5.58  | < 0.001 |
| Ulcerative colitis |            |            |         |
| Sex (female)       | 3.12       | 1.58-6.18  | 0.001   |
| Corticosteroids    | 3.62       | 1.22-10.67 | 0.02    |
| Elevated CRP       | 2.03       | 0.96-4.26  | 0.062   |
| Disease extension  | 3.79       | 1.51-9.55  | 0.005   |

IBD: Inflammatory bowel disease; E: Disease extension (for ulcerative colitis); E1: Ulcerative proctitis; E2: Left-sided ulcerative colitis; E3: Extensive disease; AZA: Azathiopurine; MTX: Methotrexate; IFX: Infliximab; ADA: Adalimumab.



**Figure 1** Types of anemia seen in inflammatory bowel disease. IBD: Inflammatory bowel disease; IDA: Iron deficiency anemia; CDA: Chronic disease anemia; UC: Ulcerative colitis.

were diagnosed with anemia received treatment. Of the patients with IDA, 23% received oral iron therapy and 40.3% received parenteral iron preparations. Of those with B<sub>12</sub>/folic acid anemia, 53.3% received B<sub>12</sub>/folic acid treatment. None of the patients in the current study received blood transfusions or were given erythropoiesis stimulant agents.

## DISCUSSION

Anemia is the most common extraintestinal finding in IBD. While anemia significantly impairs quality of life, the majority of IBD patients with anemia are not aware that some of their symptoms and/or complaints may be related to their anemia. In our current study, 52% of the IBD patients had anemia, and the rate of anemia was higher in CD patients compared to UC patients. These results are similar to those of previous studies<sup>[4,5,18,19]</sup>. In their population-based study including 756 patients (235 CD and 519 UC), Hoivik *et al.*<sup>[18]</sup> found that 48.8% of CD

patients and 20.2% of UC patients were diagnosed with anemia. Another population-based study<sup>[6]</sup> reported that among 749 IBD patients, 30% had anemia, and the rate was higher in CD patients (42%) compared to UC patients (24%). Another study conducted in Spain<sup>[19]</sup> revealed a similar incidence of anemia in IBD (41.2%). Half of the CD patients had anemia, and only one third of the UC patients were anemic. In a study conducted in Turkey<sup>[20]</sup>, the anemia rate was found to be 22% in UC and 24% in CD among 398 patients with IBD. The higher rate of anemia observed repeatedly in CD may be explained not only by increased bleeding, but also by additional mechanisms such as systemic inflammation (which can be more severe in CD) and decreased iron absorption (due to involvement of the proximal gastrointestinal tract). However, it is important to note that some studies have reported no difference in anemia rates between IBD sub-types<sup>[21]</sup>.

In our patient population, the severe anemia (Hb < 10 g/dL) rate was significantly higher in CD patients

compared to UC patients. However, Lucendo *et al.*<sup>[19]</sup> reported a lower rate of severe anemia, and that the rate of severe anemia between CD and UC was not significantly different. Another study conducted in Korea reported that the anemia rate in UC was 36.3% (similar to that of Western countries), while the rate in CD patients was 41.6%; however, the severe anemia rates between CD and UC were not significantly different<sup>[22]</sup>.

While the underlying cause of anemia in IBD patients is most likely multi-factorial, the most common causes are IDA and CDA. Multivariate analyses from published studies have repeatedly shown that being female<sup>[5,23,24]</sup> and disease activity<sup>[18,25]</sup> are the most important determining factors that increase the frequency of anemia in both UC and CD. Our results are consistent with these previous studies. The fact that anemia is more common among young women could be related to blood loss during menstrual cycles, gestation, and lactation.

In our current study, anemia was detected at a higher rate in those with active disease (CRP levels higher than 5 mg/L) than in those without. Moreover, CRP level is suggested to be the predictive factor for unresponsiveness to oral iron therapy. Therefore, intravenous iron therapy is proposed to be the first line therapy in active disease<sup>[26]</sup>. Similar to our current study, previous reports have shown that the frequency of anemia increases with increased clinical activity in IBD. These data are supported by the results of the current study indicating a high incidence of anemia in patients taking corticosteroids or immunomodulator treatment due to active disease. Immune system activation and disease-related lesions in the gastrointestinal tract have been shown to contribute to the association between disease activity and anemia. It should be kept in mind that anemia is sometimes seen in IBD patients who are in remission (rate of 18%), and therefore, these patients should be evaluated for anemia as well<sup>[24]</sup>.

Results of the current study revealed no difference in the frequency of anemia in CD patients with regards to disease behavior. However, in UC the rate of anemia was significantly increased in parallel with disease duration. The increased rate of anemia in the more extensive disease may be a result of both increased blood loss and increased burden of inflammation.

Smoking has been shown to have a low risk for causing anemia in IBD patients. This is due to these patients often developing compensatory polycythemia due to increased carbon monoxide consumption<sup>[18,24]</sup>. Previous studies have reported that while smoking is a risk factor for anemia in CD, it is a protective factor in UC<sup>[19]</sup>. Our current study did not elucidate the role of smoking in the development of anemia in IBD patients.

Although it is known that the incidence of anemia is higher in hospitalized patients than in outpatients, we could not perform a robust statistical comparison in our current study due to the low the number of hospitalized patients. In addition, the current study did not establish a significant relationship between surgery and frequency of anemia. It has been reported that the incidence of

anemia decreases as IBD disease duration progresses. However, similar to the study of Koutroubakis *et al.*<sup>[5]</sup>, results of our current study indicate no relationship between disease duration and anemia.

In our current study, the most common cause of anemia was IDA. Similar to a previously published review<sup>[7]</sup>, results of our current study reveal that IDA was more frequent in CD (32%) than in UC (26%). IDA can develop due to intestinal bleeding, dietary restrictions, or malabsorption<sup>[27]</sup>. Pro-inflammatory cytokines, such as IL-6 and bone morphogenetic protein, are increased in the circulation in active IBD. This causes increased secretion of hepcidin, which is produced in the liver and responsible for the absorption of iron. Increased hepcidin levels may cause the degradation of ferroportin channels, which allow iron to be transferred through the basolateral membrane of enterocytes, thus causing malabsorption of iron. In addition, it is known that iron accumulates in macrophages and monocytes. Basseri *et al.*<sup>[28]</sup> revealed that hepcidin expression increases in parallel with increased levels of IL-6 in CD. Concordant with this finding, Semrin *et al.*<sup>[29]</sup> showed that intestinal iron absorption is decreased in active CD compared to patients in remission.

While the diagnosis of IDA is routinely made via low serum ferritin levels (< 30 ng/mL) as well as a decrease in the TS and MCV indices, these criteria may not be valid in IBD patients, since ferritin is an acute phase reactant. When evaluating IBD patients, TS and disease activity status should always be considered along with ferritin levels<sup>[25]</sup>. In our current study, no difference was observed between CD and UC in terms of serum iron levels and mean TS. However, serum ferritin and CRP were significantly higher in CD ( $P = 0.001$ ) (Table 3).

CDA is the second most common type of anemia in IBD patients. In our current study, CDA was seen at a rate of 8%, while IDA was seen at a rate of 3.4%. In IBD patients, anemia often develops due to increased levels of cytokines [e.g., TNF- $\alpha$ , IL-1, interferon (IFN)- $\gamma$ ] and hepcidin, and decreased levels of erythropoietin<sup>[30,31]</sup>. IFN- $\gamma$  is known to inhibit the development of erythroid progenitor cells and enhance erythrocyte destruction in the spleen<sup>[32]</sup>, while TNF- $\alpha$  causes increased apoptosis in the erythroid progenitor leading to anemia<sup>[33]</sup>.

In CD, vitamin B<sub>12</sub> and folic acid deficiency due to malabsorption is reported to be 29%-33%<sup>[34]</sup>. However, in our current study, this rate was estimated to be 19%. Anemia in CD patients can be caused by dietary restrictions, malabsorption due to ileal inflammation, bacterial overgrowth, fistula development, and/or surgical resection. In UC, vitamin B<sub>12</sub> deficiency has been reported at a rate of 16%, which is lower than that reported for CD<sup>[34]</sup>. According to a study conducted in Turkey, there is a higher rate of vitamin B<sub>12</sub> deficiency in CD than UC<sup>[35]</sup>. In our current study, the rate of anemia due to vitamin B<sub>12</sub> or folic acid deficiency in UC patients was 7%. The responsible mechanisms in this situation may include ileal dysfunction following proctocolectomy and ileal pouch-anal anastomosis, bacterial overgrowth,

and reduced intestinal transit time<sup>[36]</sup>.

Anemia may sometimes occur as a side effect of drugs used for the treatment of IBD. Anemia has been reported to occur at a rate of 3% per year due to the toxic effects of thiopurines on bone marrow<sup>[37]</sup>. Other reports indicate that sulfasalazine is rarely associated with reduced folate absorption, and does not often cause anemia due to aplasia or hemolysis<sup>[38]</sup>. Results of the current study indicate a 64% anemia rate among AZA users; this is often due to vitamin B<sub>12</sub> and folic acid deficiency. The combination of IDA and megaloblastic anemia caused by thiopurines may present as normocytic normochromic anemia. Testa *et al.*<sup>[23]</sup> observed only two UC patients had autoimmune hemolytic anemia due to antibody development caused by cross-reaction against erythrocytes as a result of AZA and INF administration. In the current study, we found no evidence of drug-related hemolytic anemia in our patients.

It is recommended that IBD patients with active inflammation (high inflammatory bio-markers or endoscopic evidence due to disease activity) be evaluated for signs of anemia at least once every three months, while those in remission should be checked every six months. If the patient has anemia, further tests should be performed to determine etiology. Regular follow-up is recommended, as there is a risk of B<sub>12</sub> or folic acid deficiency in patients with small bowel disease or history of resection<sup>[12]</sup>. In our current study, further examinations were not performed in 15% of patients during follow-up, and therefore the etiology of anemia in these patients remains unknown.

Unfortunately, many clinicians still believe in the concept of asymptomatic anemia, in which anemia in IBD slowly progresses and may resolve once patients adapt to low Hb levels. In these cases, anemia is often not treated until it is severe. Only half of the patients in our follow-up were treated for anemia, which is better than the rates of previous studies.

In their 2013 study including gastroenterologists from nine European countries, Stein *et al.*<sup>[8]</sup> reported that patients with IBD were not adequately monitored and treated for anemia. The diagnosis of anemia was made based on Hb levels in 88% of patients, serum ferritin levels in 75%, and on TS in 25%. Iron deficiency (ferritin < 30 ng/mL) was detected in 76% of the patients in that study, and only 28% of them were prescribed IV iron therapy.

Danese *et al.*<sup>[1]</sup> reported that only 33% of IBD patients with anemia were being treated despite having been diagnosed. A recent study including 55 German gastroenterology centers reported that only 43.5% of IBD patients with anemia were treated, and 56% of those had received oral iron therapy<sup>[39]</sup>. In our current study, IV iron therapy was prescribed more often than oral iron therapy.

A sufficient response in the treatment of IDA after four weeks of treatment is indicated by an increase of 2 g/dL Hb or a > 30% increase in TS<sup>[40]</sup>. The target ferritin level in IV iron therapy is 400 µg/L. Oral iron

replacement therapy alone is typically only successful in cases with mild disease activity and mild anemia<sup>[41]</sup>. Due to its low cost and safety, oral iron replacement therapy is usually used by clinicians as a first line treatment. However, oral iron therapy has been associated with some side effects and mucosal injury events, and therefore the efficacy and tolerability of this therapy must be monitored during treatment<sup>[42]</sup>. In animal studies, oral and rectal iron administration has been shown to increase disease activity because they increase pro-inflammatory cytokines, such as IL-1, IL-6, TNF- $\alpha$ , and IFN- $\alpha$ <sup>[43]</sup>. Therefore, IV iron therapy is the preferred treatment for IBD patients, especially those with severe anemia who have had an inadequate response to oral iron therapy or cannot tolerate oral iron therapy<sup>[44]</sup>. The benefits of IV iron therapy include quicker improvement of iron deficiency, quicker alleviation of patients' complaints, and higher satisfaction rate from patients. Moreover, it has been shown that IV-administered iron has no effect on disease activity<sup>[45]</sup>. Since IDA has a tendency to recur in IBD, maintenance treatment should be continued for at least three months<sup>[46]</sup>. In cases of unresponsiveness to all types of anemia therapies, the patient should be referred to a hematologist<sup>[40]</sup>.

In IBD patients with anemia, it is of utmost importance to treat the underlying cause(s) and to control inflammation. Over time, attacks of inflammation in IBD lead to decreased iron absorption. In addition, TNF- $\alpha$  is known to increase bone marrow suppression. In our current study, there was a lower rate of anemia among patients undergoing anti-TNF therapy compared to other treatment groups. Anti-TNF therapy is becoming an increasingly preferred treatment. These patients had a lower rate of anemia, which may be related to the fact that anti-TNF agents suppress adverse effects on bone marrow, decrease inflammation, and provide effective mucosal improvement. Similarly, another study reported that anti-TNF therapy improved anemia by controlling inflammation and disease activity in IBD patients<sup>[47]</sup>. It has been suggested that anti-TNF therapy regulates erythropoiesis at various levels. However, some studies have shown that the prevalence of anemia was higher in patients treated with anti-TNF agents than those treated with other drugs (e.g., immunomodulators, corticosteroids, aminosalicylates)<sup>[48]</sup>. The higher prevalence reported in those studies is associated with the fact that these drugs (anti-TNF agents) are often used in patients with increased disease severity.

One of the limitations of our current study is that it was retrospective. Therefore, we did not have any information about disease activity or smoking status. However, since we could not obtain or confirm the accuracy of the patients' clinical activity indices, we utilized CRP levels to interpret disease activity. Moreover, we could not evaluate the etiology of anemia in 15.3% of our patients because their anemia parameters were not evaluated during follow-up. In addition, we did not separately evaluate the anemia rates of pediatric-



onset IBD and adult-onset IBD, and therefore we could not compare the frequency of anemia between these groups. Further, we did not evaluate anemia-associated symptom rates or quality of life in this study. Lastly, the current study may not represent the general Turkish patient population as it was conducted with IBD patients with more severe and problematic conditions who were being followed at a tertiary referral university hospital.

In conclusion, because almost half of IBD patients have anemia and anemia causes a multitude of negative effects on patients, its presence should be further examined, and if necessary treated with regards to disease activity. It should be kept in mind that the most common cause of anemia is iron deficiency. Being female and disease activity are important risk factors in the development of anemia, and disease involvement is an additional factor in UC. Treatment rates should be increased in IBD patients with anemia. To conclude, anemia should be recognized, investigated, and treated in IBD patients.

## ARTICLE HIGHLIGHTS

### Research background

Inflammatory bowel disease (IBD) is a chronic idiopathic disease with a relapsing and remitting course. The most common extraintestinal finding seen in IBD patients is anemia, which decreases both the quality of life and the ability to work. The first study to report the incidence of anemia in Turkish patients with IBD reported that 58.2% had anemia at least once during an 18-year follow-up period.

### Research motivation

The prevalence of anemia is higher in IBD patients than in the general population. The most common causes of anemia in IBD are iron deficiency anemia (IDA) and chronic disease anemia (CDA). Although IBD-related anemia has a relatively high prevalence, its diagnosis and treatment is generally overlooked.

### Research objectives

The current study aimed to determine the frequency and types of anemia in IBD patients, to determine the relationship between anemia and disease characteristics, and to determine the most effective treatment approach.

### Research methods

This study retrospectively evaluated 465 patients who were diagnosed with IBD and followed-up between June 2015 and June 2016 in the Gastroenterology/IBD outpatient clinic or ward of Dokuz Eylül University, Medical Faculty Hospital. The IBD diagnoses were made in accordance with the new European Crohn's and Colitis Organization (ECCO) guidelines, and were confirmed according to standard clinical, endoscopic, histologic, and radiological criteria. Demographic and clinical data as well as endoscopic activities were obtained from hospital records. We used the World Health Organization guidelines to diagnose anemia in our IBD patients. Males were diagnosed with anemia if they had hemoglobin values less than 13 g/dL, and females were diagnosed if they had hemoglobin values less than 12 g/dL. Severe anemia was defined as having Hb values below 10 g/dL for both sexes. We evaluated the lowest hemoglobin levels of each patient during follow-up, as well as iron levels and other anemia parameters. Three main classifications of anemia were selected in accordance with the European consensus on anemia in IBD, including IDA, CDA, and mixed anemia.

### Research results

This study included the data from 465 IBD patients (54.6% male and 45.4%

female) who were newly diagnosed or were being followed-up with in our hospital. Of these patients, 55.3% were diagnosed with CD and 44.7% with UC. Approximately fifty-two percent of the IBD patients had anemia. Anemia frequency was higher in CD than in UC. Anemia was more common among women than men. Severe anemia was observed in 21.6% of patients with CD and 9.8% of patients with UC. IDA was the most common type of anemia (29.9%).

Approximately 50.4% of all of the IBD patients who were diagnosed with anemia in this study received treatment. Of the patients with IDA, 23% received oral iron therapy and 40.3% received parenteral iron preparations. Of those with B<sub>12</sub>/folic acid anemia, 53.3% received B<sub>12</sub>/folic acid treatment. None of the patients in the current study received blood transfusions or were given erythropoiesis stimulant agents.

### Research conclusions

Since almost half of IBD patients have anemia, and because anemia causes a multitude of negative effects on patients, its presence should be further examined, and if necessary, treated with regards to disease activity. It should be kept in mind that the most common cause of anemia is iron deficiency. Treatment rates should be increased in IBD patients with anemia.

### Research perspectives

Anemia in IBD patients must be monitored throughout active and remissive disease and treated accordingly.

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## Prospective Study

# Low-dose spectral insufflation computed tomography protocol preoperatively optimized for T stage esophageal cancer - preliminary research experience

Yue Zhou, Dan Liu, Ping Hou, Kai-Ji Zha, Feng Wang, Kun Zhou, Wei He, Jian-Bo Gao

Yue Zhou, Ping Hou, Kai-Ji Zha, Jian-Bo Gao, Department of Radiology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan Province, China

Dan Liu, Department of Gastroenterology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan Province, China

Feng Wang, Wei He, Department of Oncology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan Province, China

Kun Zhou, Department of Thoracic Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan Province, China

ORCID number: Yue Zhou (0000-0001-7781-7228); Dan Liu (0000-0003-3081-792X); Ping Hou (0000-0002-3932-8789); Kai-Ji Zha (0000-0002-8774-0698); Feng Wang (0000-0002-1607-6478); Kun Zhou (0000-0002-2234-3069); Wei He (0000-0001-5214-5162); Jian-Bo Gao (0000-0003-1252-7144).

**Author contributions:** Gao JB and Zhou Y designed the research; Zhou Y, Hou P, Zha KJ, Liu D, Zhou K and He W performed the research and data analysis; Zhou Y, Hou P and Zha KJ contributed to the statistical analysis; Liu D, Zhou K and He W collected the data and assigned the forms; Zhou Y and Gao JB wrote the paper.

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**Correspondence to:** Jian-Bo Gao, MD, PhD, Professor, Department of Radiology, The First Affiliated Hospital of Zhengzhou University, No. 1 East Jianshe Road, Zhengzhou 450052, Henan Province, China. [cjr\\_gaojianbo@163.com](mailto:cjr_gaojianbo@163.com)  
**Telephone:** +86-371-67966890  
**Fax:** +86-371-66970906

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## Abstract

### AIM

To evaluate the T stage of esophageal squamous cell carcinoma (ESCC) using preoperative low-dose esophageal insufflation computed tomography (EICT).

### METHODS

One hundred and twenty ESCC patients confirmed by surgery or esophagoscopy were divided into three groups. Groups B and C were injected with 300 mgI/kg contrast medium for automatic spectral imaging assist



(GSI assist), while group A underwent a conventional 120 kVp computed tomography (CT) scan with a 450 mgI/kg contrast medium injection. EICT was performed in group C. Group A was reconstructed with filtered back projection, and groups B and C were reconstructed with 50% adaptive statistical iterative reconstruction. The contrast-to-noise ratio of lesion-to-mediastinal adipose tissue and the radiation dose were measured. Specific imaging features were observed, and T stage ESCCs were evaluated.

## RESULTS

The sensitivity and accuracy of the T1/2 stage were higher in group C than in groups A and B (sensitivity: 43.75% *vs* 31.82% and 33.33%; accuracy: 54.29% *vs* 46.67% and 52.50%, respectively). With regard to the T3 stage, the sensitivity and specificity in group C were higher than those in groups A and B (sensitivity: 56.25% *vs* 41.17% and 44.44%; specificity: 73.68% *vs* 67.86% and 63.64%, respectively). The diagnostic sensitivity, specificity and accuracy of the T4 stage were similar among all groups. There were no significant differences in volume CT dose index [ $(5.91 \pm 2.57)$  mGy *vs*  $(3.24 \pm 1.20)$  *vs*  $(3.65 \pm 1.77)$  mGy], dose-length product [ $(167.10 \pm 99.08)$  mGy·cm *vs*  $(113.24 \pm 54.46)$  mGy·cm *vs*  $(117.98 \pm 32.32)$  mGy·cm] and effective dose [ $(2.52 \pm 1.39)$  *vs*  $(1.63 \pm 0.76)$  *vs*  $(1.73 \pm 0.44)$  mSv] among the groups ( $P > 0.05$ ). However, groups B and C received similar effective doses but lower iodine loads than group A [(300 *vs* 450) mgI/kg].

## CONCLUSION

EICT combined with GSI assist allows differential diagnosis between the T1/2 and T3 stages. The ability to differentially diagnose the T3 and T4 stages of medullary ESCC can be improved by quantitatively and qualitatively analyzing the adipose tissue in front of the vertebral body.

**Key words:** Esophageal neoplasms; tomography; tumor staging

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**Core tip:** Esophageal insufflation computed tomography (EICT) is a method of insufflating air into the stomach before computed tomography examination, which fully expands the esophageal lumen. The optimal monochromatic energy level clearly displays esophageal lesions and surrounding adipose infiltration by means of effectively improving the image quality and resolution. Our study demonstrates that EICT combined with GSI assist technology contributes to better performance in the differential diagnosis between the T1/2 *vs* T3 stages and the T3 *vs* T4 stages in medullary esophageal cancer.

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## INTRODUCTION

Esophageal carcinoma (EC) is a common malignant tumor of the digestive system. Mortality due to EC is approximately 300000 people per year worldwide<sup>[1]</sup>. The most common pathology subtype in Asia is esophageal squamous cell carcinoma (ESCC). As it is rare for patients to exhibit early symptoms of ESCC, patients typically receive treatment in the mid- and late stages (T3/4, N+ or M1)<sup>[2]</sup>. The overall five-year survival rate of progressive ESCC (T3/4 or N+) is only 42% after surgical resection or preoperative neoadjuvant chemotherapy<sup>[3]</sup>. Identification of the correct T stage of ESCC by preoperative imaging plays a critical role in the development, treatment and prognosis of patients.

Endoscopic ultrasonography (EUS) can be used clinically to determine the infiltration of ESCC and the possibility of surgical resection. However, the detection range is limited to centimeters from the center of the ultrasonic probe without interference or severe stenosis. Griffin *et al*<sup>[4]</sup> reported that inflammation or fibrous tissue surrounding ESCC tissue leads to over-staging of the local T stage. Currently, computed tomography (CT) and positron emission tomography/computed tomography (PET/CT) are common methods used to evaluate the T stage before ESCC treatment<sup>[5-7]</sup>. Variations in the sensitivity and specificity of these common methods is 27%-67% and 33%-93%, respectively<sup>[8,9]</sup>. Some studies continue to use traditional CT enhancement with low spatial and density resolutions. Konieczny *et al*<sup>[10]</sup> asserted that the accuracy of traditional 64-slice CT enhancement was 34% for EC. The sensitivity and specificity of CT or PET/CT are approximately 31% and 59% for diagnosis of the T1/2 stage, 60% and 64% for diagnosis of the T3 stage, and 100% and 4% for diagnosis of the T4 stage, respectively, which are not satisfactory.

Conventional CT has limitations for ESCC staging or restaging after treatment. Esophageal insufflation CT (EICT) is a method of insufflating air into the stomach before CT examination, which fully expands the esophageal lumen<sup>[11]</sup>. Diagnosis of the T1 or T2 stage has low accuracy because of the difficulty in visualizing the esophageal mucosa<sup>[12,13]</sup>. The optimal monochromatic energy level clearly displays esophageal lesions and the surrounding adipose infiltration by effectively improving the image quality and resolution. The optimal monochromatic energy level can be used for diagnosis, treatment selection, and therapeutic monitoring. Hence, we aimed to evaluate the T stage of ESCC using low-dose spectral insufflation CT, and we

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discuss the accuracy of this technique for diagnosing the T stage preoperatively.

## MATERIALS AND METHODS

### Subject enrollment

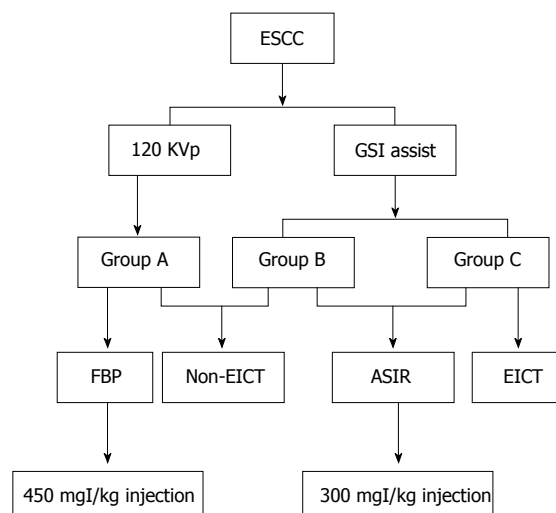
This study was approved by the Institutional Review Board. All patients enrolled in this study provided informed consent.

In this single-institution study, 120 patients with a biopsy-proven esophageal malignancy who were being considered for radical treatment and who had already undergone EUS with a median age of 58 years (range, 48-83 years) were recruited from November 2015 to August 2017. The patients included 66 males and 54 females, with a median age of 55.4 years (range, 48-83 years). The typical clinical symptoms included vomiting, progressive dysphagia, intermittent sternal sensation, hematemesis and a sense of frustration. All patients considered for radical treatment were staged according to spectral CT and EUS within 6 wk.

The exclusion criteria included the following: (1) patients with esophageal cancer undergoing spectral CT to detect recurrence; (2) patients with a poor physical condition or a combination of severe heart, liver or kidney dysfunction; (3) patients with a history of iodine allergy, making them unsuitable for enhanced examination; and (4) patients with a history of other cancers.

### Spectral CT protocol and data acquisition

Patients were divided into three groups that included 45 patients (group A), 40 patients (group B) and 35 patients (group C). Patients were required to fast for 6 h prior to the investigation and were administered an intramuscular injection of amidoamine (20 mg) 10-15 min before experimental procedures. EUS was performed using a PHILIP IU22 Color Doppler Diagnostic Apparatus (Philip, Eindhoven, The Netherlands) with a 5-10 Hz radical or linear endoscope. Then, a dual-phase contrast enhancement spectral spiral CT was performed with a spectral CT scanner (Discovery CT, GE Healthcare, Waukesha, WI, United States) from the thoracic inlet to the bottom of the lungs. The imaging parameters for group B were as follows: tube voltage: 80 kV and 140 kV with a fast kV-switching technique; tube current: auto mA with a slice thickness of 5 mm. Iobitrido (Guerbet, Paris, French), containing 350 mg/mL of iodine, was injected at a dose of 300 mgI/kg. The injection rate was calculated as the weight in kilograms divided by 30 s. A triggering scan was performed when the CT attenuation of the aortic arch reached the level of 100 HU. The starting time was 90 s after triggering. The saline tracer injection rate was similar to that of the contrast medium. For group A, conventional 120 kVp chest-enhanced CT scanning was performed with an injection dose of 450 mgI/kg. The remaining parameters were similar to those of group B.



**Figure 1** Study flow chart. ESCC: Esophageal squamous cell carcinoma; FBP: Filtered back projection; EICT: Esophageal insufflation computed tomography; ASIR: Adaptive statistical iterative reconstruction.

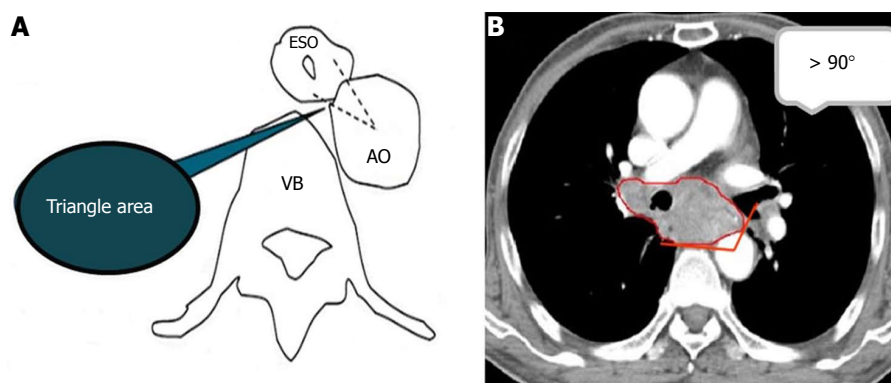
For group C, a gastric tube was inserted into the stomach *via* the nasal cavity 10-15 min before the CT examination. The depth of insertion was referenced to the location of the esophageal lesion, and the end of the tube was fixed near the nostrils. Patients were asked to press a balloon to fill the stomach with air. Pressure was maintained between 4-4.67 kPa. Patients were required to keep their lips tightly closed and to fill the esophagus as much as possible during the process of filling. The rest of the parameters were similar to those of group B.

### Qualitative and quantitative analyses

CT images (40-140 keV, monochromatic) were reconstructed using spectral imaging analysis software (GE Healthcare, Waukesha, WI, United States). The 50% adaptive statistical iterative reconstruction (ASIR) algorithm and standard filtered back projection reconstruction were applied to the decomposition images of group A and groups B and C, respectively. A flowchart of the study procedures is shown in Figure 1.

### Special CT features observation

Two radiologists (Zha KJ and Zhou Y) with ten years of experience in CT diagnosis independently observed and recorded the special features of the CT images in a blinded and randomized manner using a dedicated workstation (Advantage Workstation 4.6, GE Healthcare, Waukesha, WI, United States). In the case of a discrepancy between interpretations, a consensus was reached by discussion. The main observations included enhanced features for ESCC (layered/unlayered enhancement) and morphological changes near ESCC tissue (a triangle area in front of the vertebral body, trachea, bronchus and aorta). A layered enhanced feature for ESCC was used to clearly identify the layers of the esophageal wall to effectively determine infiltration.



**Figure 2 The morphological features of the triangular area in front of the vertebral body.** A: Sketch of the triangular area. The triangular space is surrounded by the outer esophageal membrane, thoracic aorta and vertebral body. B: The contact arc between the tumor and thoracic aorta is less than 45°; a contact arc of 45-90° corresponds to suspected invasion, and a contact arc larger than 90° indicates thoracic aorta invasion. VB: Vertebral body.

As described in Figure 2A, the triangular area in front of the vertebral body is surrounded by the outer esophageal membrane, the thoracic aorta and the vertebral body. Under normal circumstances, this area is filled with adipose tissue; however, when invaded by ESCC, this area is blurred or disappears. The narrow space between the anterior wall of the esophagus and the trachea is connected by loose connective tissue. When the tracheal bronchus is invaded, the space is depicted as having an ill-defined boundary or tracheal bronchus deformation and displacement. In general, the contact arc between the tumor and the thoracic aorta is less than 45°, while an arc of 45°-90° indicates invasion, and an arc larger than 90° indicates thoracic aorta invasion (Figure 2B).

### Subjective imaging evaluation

From the monochromatic images, an analysis was performed to obtain the optimal energy level to provide the best contrast-to-noise ratio (CNR) between ESCC tissue and surrounding adipose tissue. Selected circular or oval-shaped areas from 70-80 mm<sup>2</sup> were used for the regions of interest (ROI) measurement, which contained ESCC tissue and surrounding adipose tissue. The GSI Viewer software package automatically calculated the best CNR values from 101 sets of monochromatic images. The standard deviation (SD) of adipose tissue inside the mediastinal space at the same level represents image noise. The ROI was placed in the region as homogeneously as possible (an average of three ROIs). CNR was calculated using the following formula:  $CNR = (CT_{ESCC} - CT_{adipose}) / SD_{adipose}$ . The normalized iodine concentration was obtained by dividing the iodine concentration (IC) for ESCC tissue ( $IC_{ESCC}$ ) by that for the aorta ( $IC_{aorta}$ ). The normalized iodine concentration (NIC) was calculated as  $NIC = IC_{ESCC} / IC_{aorta}$ .

### Radiation dose

The volume CT dose index (CTDI<sub>vol</sub>, mGy) and dose-length product (DLP, mGy·cm) in the dose report were also recorded. The estimated effective dose (ED, mSv)

was calculated by multiplying the DLP by 0.014 (as recommended by the International Commission on Radiological Protection (ICRP) for chest CT examinations).

### Pathological subtype and T stage

The spectral CT results were compared with the results of other combined staging investigations, such as EUS and PET. For unresectable disease, sections were obtained for histological assessment, additional imaging [PET/CT, magnetic resonance imaging (MRI)] or clinical course determination, such as rapidly progressive disease or response to treatment. For potentially resectable disease, lesion sections were taken and frozen at the time of resection when appropriate, and information was obtained upon subsequent relapse and survival.

The T stage was reported according to the maximum wall thickness of ESCC tissue using the criteria of the classification system by Konieczny *et al.*<sup>[10]</sup> and Jones *et al.*<sup>[14]</sup>, and consistent with the 7<sup>th</sup> TNM edition<sup>[4,15]</sup>. The T status for CT diagnosis was defined as follows: the T1 and T2 stages were combined because it was impossible to differentiate between the esophageal wall layers on MDCT images. The T1/2 stage was defined as a tumor wall thickness of at least 5-10 mm without evidence of mediastinal involvement. The T3 stage was defined as a tumor wall thickness greater than 10 mm with mediastinal involvement but no invasion of adjacent structures. The T4a (invasion of pleura, pericardium and diaphragm) and T4b (invasion of other structures, *e.g.*, aorta, vertebral body and trachea) stages were defined as a tumor wall thickness greater than 10 mm and invaded adjacent structures.

The pathological subtype was classified based on the advanced esophagus cancer pathology classification criteria of the NCCN guidelines (2017. V3). The pathological subtypes included medullary type (wall thickness with symmetry or partial lumen stenosis), mushroom type (wall thickness similar to a flat mushroom mass), ulcer type (a larger and deeper ulcer on the surface of the wall) and narrowing type (narrow and obstructed lumen with a dilated upper segment).

**Table 1 Basic clinical characteristics of patients *n* (%)**

| Variable                             | Group A ( <i>n</i> = 45) | Group B ( <i>n</i> = 40) | Group C ( <i>n</i> = 35) |
|--------------------------------------|--------------------------|--------------------------|--------------------------|
| Gender                               |                          |                          |                          |
| Male                                 | 25 (56)                  | 19 (48)                  | 22 (64)                  |
| Female                               | 50 (44)                  | 21 (52)                  | 13 (36)                  |
| Age (yr)                             |                          |                          |                          |
| Medium                               | 67                       | 61                       | 63                       |
| Range                                | 55-82                    | 52-79                    | 50-81                    |
| Location                             |                          |                          |                          |
| Upper esophagus                      | 11 (24)                  | 8 (20)                   | 6 (16)                   |
| Middle esophagus                     | 14 (32)                  | 11 (28)                  | 11 (32)                  |
| Lower esophagus                      | 20 (44)                  | 22 (56)                  | 18 (52)                  |
| Differentiation degree               |                          |                          |                          |
| High                                 | 17 (38)                  | 9 (23)                   | 10 (28)                  |
| Medium                               | 23 (50)                  | 25 (62)                  | 14 (40)                  |
| Low                                  | 5 (12)                   | 6 (15)                   | 11 (32)                  |
| Symptom                              |                          |                          |                          |
| Progressive dysphagia                | 18 (40)                  | 19 (48)                  | 21 (60)                  |
| Vomiting                             | 13 (28)                  | 8 (20)                   | 4 (12)                   |
| Intermittent sternal sensation       | 9 (20)                   | 8 (20)                   | 6 (16)                   |
| Hematemesis and sense of frustration | 5 (12)                   | 5 (12)                   | 4 (12)                   |

### Statistical analysis

The Statistical Package for the Social Sciences version 19.0 software program (SPSS, Inc., Chicago, IL, United States) was used for statistical analyses. Quantitative variables are expressed as the mean  $\pm$  SD. Paired *t*-tests were used to compare age, BMI and radiation dose among the image reconstruction protocols. One-way analysis of variance was used to compare objective image noise. The least significant difference correction was used for multiple comparisons. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy for determining the T stage of the three groups were calculated, and the positive predictive value and negative predictive value were calculated. Inter-observer agreements between the two radiologists were based on the percentage agreement and simple Cohen's kappa statistic. The significance level for all tests was 5% (two-sided).

## RESULTS

The basic characteristics of the patients are summarized in Table 1. No significant differences were found in gender, tumor location, differentiation, and clinical symptoms among groups ( $P < 0.05$  for all). The medullary type comprised the largest proportion in each group, followed by the mushroom and ulcer types; the narrowing type corresponded to the smallest proportion.

### Special CT features for T1/2 and T3 stage differentiation

The proportions of layered enhancement in medullary ESCC tissue in groups A, B and C were 33%, 56% and 75% (for the T1/2 stage) and 20%, 20% and 11% (for the T3 stage), respectively; those for ulcer type were 33%, 0% and 33% (for the T1/2 stage) and 0%, 0% and 20% (for the T3 stage); and those for mushroom type were 20%, 0% and 60% (for the T1/2 stage) and

0% for all groups (for the T3 stage). The presentation of layered enhancement significantly differed between T1/2 and T3 stage medullary ESCC in group C ( $P < 0.05$ ) but not between those stages in groups A and B ( $P > 0.05$ ), and there was no significant difference between the T1/2 and T3 stages in the ulcer and mushroom types ( $P > 0.05$  for all) (Table 2, Figures 3 and 4).

### Special CT features for T3 and T4 stage differentiation

The optimal monochromatic image with the best CNR in groups B and C was mainly located at ( $50.18 \pm 2.64$ ) KeV. The CNR<sub>lesion-to-adipose</sub> at 50 keV in groups B and C was higher than that of group A ( $P < 0.05$ ); however, there was no significant difference in the CNR<sub>lesion-to-adipose</sub> between groups B and C ( $P < 0.05$ ). In terms of the morphological change of the triangular area in front of the vertebral body, the proportion of adipose blur or disappearance in groups A, B and C was 40%, 30% and 22% (for the T3 stage) and 50%, 57% and 54% (for the T4 stage) for medullary ESCC, respectively; 25%, 0% and 0% (for the T3 stage) and 0% in all groups (for the T4 stage) for the ulcer type; and 0% in all groups (for the T3 and T4 stage) for the mushroom type. There were no significant differences in morphological changes between the T3 and T4 stages for medullary type, ulcerative type and mushroom type ESCC (Table 3).

The quantitative parameters IC and NIC of adipose tissue in the triangular area in front of the vertebral body during arterial phase (AP) and venous phase showed significant differences in their ability to discriminate the T3 and T4 stages ( $P < 0.05$ ). The receiver operating characteristic curve demonstrated that the area under the curve for NIC was higher than that for IC. When the threshold of NIC during AP was -0.03, the sensitivity and specificity for identifying the T3 stage were 83.30% and 83.33%, respectively (Figure 5).

Combined analyses of the morphological features

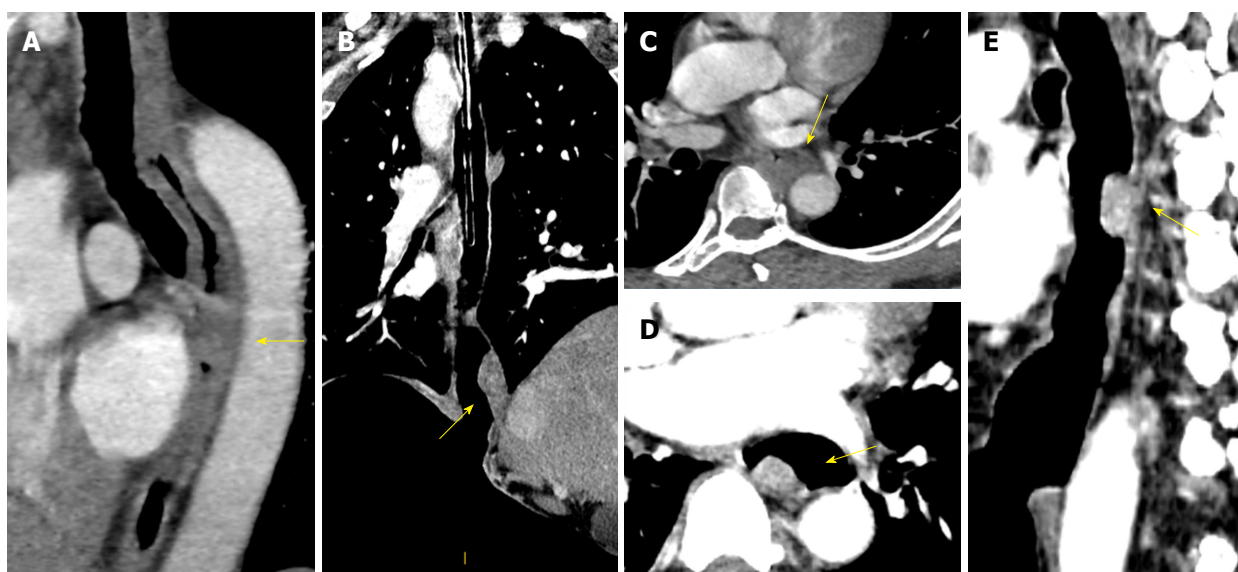


**Table 2** Comparison between esophageal squamous cell carcinoma-enhanced features and pathological T stage

| Group | Enhancement feature | Medullary type |    | Ulcerative type |    | Mushroom type |    | Total |
|-------|---------------------|----------------|----|-----------------|----|---------------|----|-------|
|       |                     | T1/2           | T3 | T1/2            | T3 | T1/2          | T3 |       |
| A     | Layered             | 3              | 2  | 2               | 0  | 1             | 0  | 8     |
|       | Unlayered           | 6              | 8  | 6               | 4  | 4             | 3  | 31    |
| Total |                     | 9              | 10 | 8               | 4  | 5             | 3  | 39    |
| B     | Layered             | 5              | 2  | 0               | 0  | 0             | 0  | 7     |
|       | Unlayered           | 4              | 8  | 2               | 5  | 4             | 3  | 26    |
| Total |                     | 9              | 10 | 2               | 5  | 4             | 3  | 33    |
| C     | Layered             | 6              | 1  | 1               | 1  | 3             | 0  | 12    |
|       | Unlayered           | 2              | 8  | 2               | 4  | 2             | 2  | 20    |
| Total |                     | 8              | 9  | 3               | 5  | 5             | 2  | 22    |

**Table 3** Comparisons between morphological changes of the triangular area in front of the vertebral body and pathological T stage

| Group | Morphological change of triangle area in front of vertebral body | Medullary type |    | Ulcerative type |    | Mushroom type |    | Total |
|-------|--|----------------|----|-----------------|----|---------------|----|-------|
|       |  | T3             | T4 | T3              | T4 | T3            | T4 |       |
| A     | Clear  | 6              | 3  | 3               | 0  | 3             | 0  | 15    |
|       | Blurred or disappeared   | 4              | 3  | 1               | 0  | 0             | 0  | 8     |
| Total |  | 10             | 6  | 4               | 0  | 3             | 0  | 23    |
| B     | Clear  | 7              | 3  | 5               | 0  | 3             | 0  | 18    |
|       | Blurred or disappeared   | 3              | 4  | 0               | 0  | 0             | 0  | 7     |
| Total |  | 10             | 7  | 5               | 0  | 3             | 0  | 25    |
| C     | Clear  | 7              | 6  | 5               | 0  | 2             | 0  | 20    |
|       | Blurred or disappeared   | 2              | 7  | 0               | 0  | 0             | 0  | 9     |
| Total |  | 9              | 13 | 5               | 0  | 2             | 0  | 29    |



**Figure 3** Typical cases showing the esophageal wall of group A, B and C. A: A sagittal reformatted image obtained via 120 kVp combined with FBP reconstruction shows unlayered enhanced esophageal cancer in a 53-year old patient (yellow arrow). The esophageal wall appears to be generally thickened over a long distance but without an enhanced layer. B: Moderately enhanced esophageal cancer in the lower esophagus of a 61-year old patient. The coronal reformatted image obtained via EICT combined with GSI assist at 50 KeV shows that the lesion appears to protrude into the lumen (yellow arrow). The lumen is filled with air without esophageal wall shrinkage. C: A 63-year old patient with histopathological T2N0M0. The axial image in the venous phase shows esophageal wall thickening, but it is difficult to identify layers (yellow arrow). Due to nose or mouth leakage, non-EICT is displayed in the venous phase for this patient. D-F: The same patient as in (C); axial and sagittal images obtained via EICT combined with GSI assist at 50 KeV show the wall thickness as if a partial mass was present with a moderate enhancement in the arterial phase (yellow arrow). EICT: Esophageal insufflation computed tomography.

and NIC during AP in the triangular area in front of the vertebral body highlighted a significant difference in discriminating T3 and T4 stage medullary ESCC in groups B and C ( $P < 0.05$ ), and there were no significant differences between T3 and T4 stage ulcer and mushroom

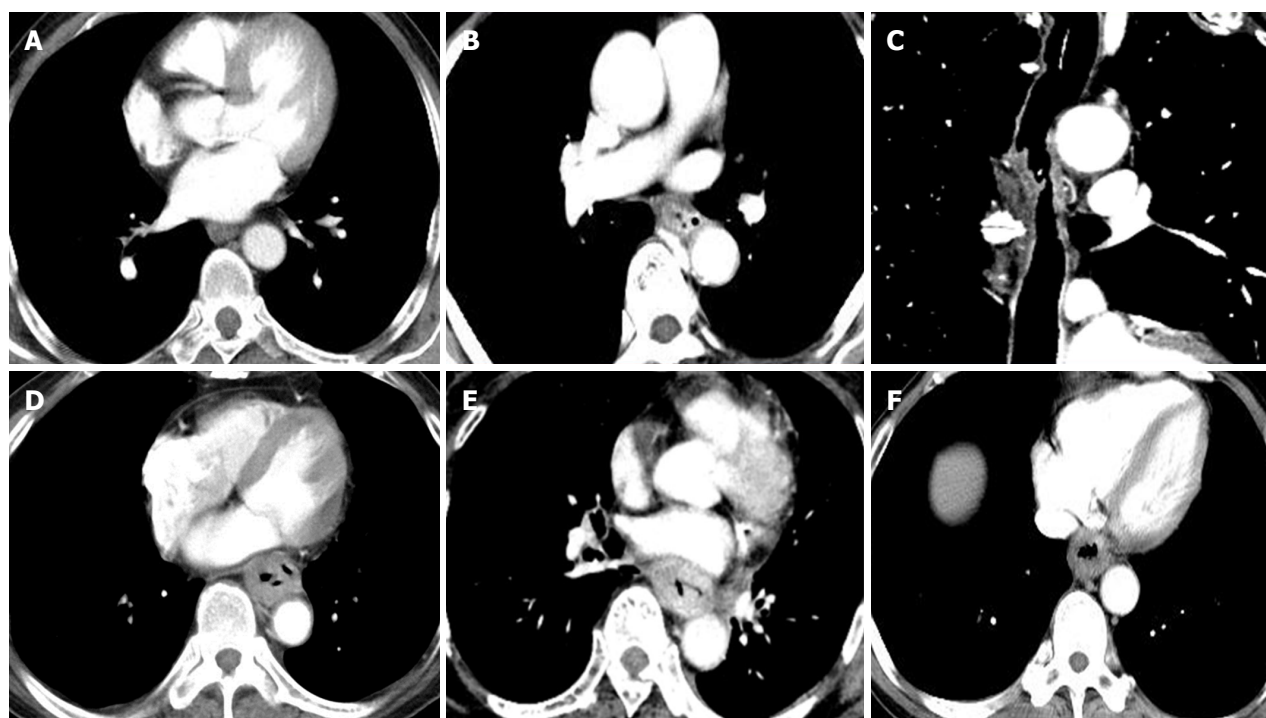
type ESCC ( $P > 0.05$  for all) (Table 4, Figure 6).

#### T stage comparisons

The sensitivity and accuracy in group C in terms of diagnosing the T1/2 stage were higher than those in the

**Table 4** Comparison of pathological T stage and combined diagnosis of the triangular area in front of the vertebral body

| Group | T stage | Medullary type |    | Ulcerative type |    | Mushroom type |    | Total |
|-------|---------|----------------|----|-----------------|----|---------------|----|-------|
|       |         | T3             | T4 | T3              | T4 | T3            | T4 |       |
| A     | T3      | 6              | 1  | 3               | 0  | 3             | 0  | 13    |
|       | T4      | 4              | 5  | 1               | 0  | 0             | 0  | 10    |
| Total |         | 10             | 6  | 4               | 0  | 3             | 0  | 23    |
| B     | T3      | 8              | 1  | 5               | 0  | 3             | 0  | 17    |
|       | T4      | 2              | 6  | 0               | 0  | 0             | 0  | 8     |
| Total |         | 10             | 7  | 5               | 0  | 3             | 0  | 25    |
| C     | T3      | 6              | 2  | 5               | 0  | 2             | 0  | 15    |
|       | T4      | 3              | 11 | 0               | 0  | 0             | 0  | 14    |
| Total |         | 9              | 13 | 5               | 0  | 2             | 0  | 29    |



**Figure 4** Typical cases between T1/2 and T3 stages of esophageal squamous cell carcinoma in group A, B and C. A: An axial image obtained via 120 kVp combined with FBP reconstruction of a 56-year old patient shows unlayered enhanced wall thickening at the lower esophagus with histopathological T2N0M0. B: A 62-year old, correctly-staged patient with histopathological T2N0M0ESCC of the middle esophagus. The axial image at 50 KeV obtained by GSI assist depicts significantly layered enhanced wall thickening, which was regarded as invasion within the submucosal or muscle layer. C: A 58-year old patient with histopathological T2N0M0; sagittal reformatted image obtained via EICT combined with GSI assist at 50 KeV shows slightly mucosal enhanced wall thickening with an ulcer on the surface. D: An axial image obtained via 120 kVp combined with FBP reconstruction of a 59-year old patient shows unlayered enhanced wall thickening at the lower esophagus with histopathological T3N1M0. E: A 55-year old patient with T3N0M0; axial image obtained using GSI assist at 50 KeV shows more obvious enhanced wall thickening than the image obtained by conventional 120 kVp. F: A 61-year old patient with T3N0M0; EICT combined with GSI assist exhibits unlayered enhanced esophageal wall thickening surrounding the air-filled lumen. EICT: Esophageal insufflation computed tomography; ESCC: Esophageal squamous cell carcinoma.

other groups. With regard to diagnosing the T3 stage, the sensitivity and specificity were higher in group C than in the other groups. The accuracy of diagnosing the T4 stage between groups was similar (Table 5).

Compared with the pathological results, mucosa enhancement was identified in 31.82% (7/22), 33.33% (5/15) and 43.75% (7/16) of cases for T1/2 stage ESCC in groups A, B and C, respectively; 68.18% (15/22), 66.67% (10/15) and 56.25% (9/16) of these cases were upstaged to T3, respectively.

There were 41.17% (7/17), 44.44% (8/18) and 56.25% (9/16) cases with T3 stage ESCC in groups A, B and C, respectively; 35.29% (6/17), 27.78% (5/18)

and 25% (4/16) of these cases were upstaged to the T4 stage. Four cases were characterized by blurring of adipose tissue between tumor and adjacent structures, seven cases showed significant enhancement of the esophageal layer, three cases had an unclear boundary mass or ulcer, and 23.53% (4/17), 27.78% (5/18) and 18.75% (3/16) cases were down-staged to T1/2.

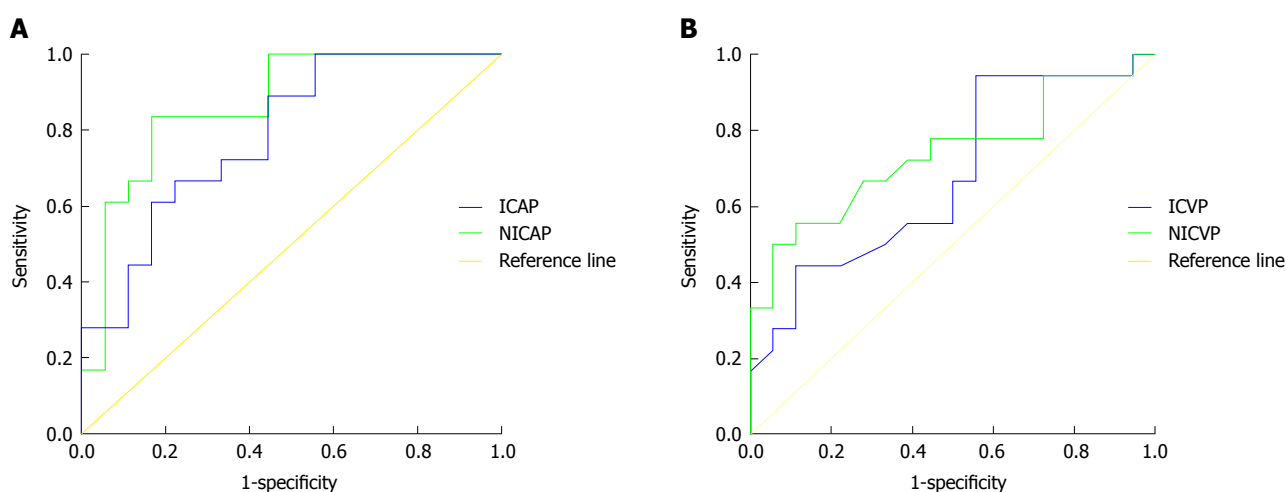
#### Radiation dose

There were no significant differences in CTDIvol [(5.91 ± 2.57) mGy vs (3.24 ± 1.20) mGy vs (3.65 ± 1.77) mGy], DLP [(167.10 ± 99.08) mGy·cm vs (113.24 ± 54.46) mGy·cm vs (117.98 ± 32.32) mGy·cm] and ED

**Table 5 Accuracy of T stage comparisons between groups**

| Stage | Group | n  | TP | TN | FP | FN | Sensitivity (%) | Specificity (%) | Accuracy (%) | PPV (%) | NPV (%) |
|-------|-------|----|----|----|----|----|-----------------|-----------------|--------------|---------|---------|
| T1/2  | A     | 45 | 7  | 9  | 15 | 14 | 31.82           | 60.87           | 46.67        | 43.75   | 48.28   |
|       | B     | 40 | 5  | 9  | 10 | 16 | 33.33           | 64.00           | 52.50        | 35.71   | 61.54   |
|       | C     | 35 | 7  | 7  | 9  | 12 | 43.75           | 63.16           | 54.29        | 50.00   | 57.14   |
| T3    | A     | 45 | 7  | 9  | 10 | 19 | 41.17           | 67.86           | 57.78        | 43.75   | 65.52   |
|       | B     | 40 | 8  | 8  | 10 | 14 | 44.44           | 63.64           | 55.00        | 50.00   | 58.33   |
|       | C     | 35 | 9  | 5  | 7  | 14 | 56.25           | 73.68           | 65.71        | 60.00   | 66.67   |
| T4    | A     | 45 | 5  | 12 | 1  | 27 | 83.33           | 69.23           | 71.11        | 29.41   | 96.43   |
|       | B     | 40 | 6  | 10 | 1  | 23 | 85.71           | 69.70           | 72.50        | 37.50   | 95.83   |
|       | C     | 35 | 11 | 7  | 2  | 15 | 84.62           | 68.18           | 74.29        | 61.11   | 94.12   |

TP: True positive; TN: True negative; FP: False positive; FN: False negative; PPV: Positive predictive value; NPV: Negative predictive value.



**Figure 5 Receiver operating characteristic curves.** Graphs showing the sensitivity and specificity of both iodine concentration and normalized iodine concentration of adipose tissue in the triangular area in front of the vertebral body during the arterial phase and the venous phase for differential diagnosis of T3 and T4 stages. AP: Arterial phase; VP: Venous phase; IC: Iodine concentration; NIC: Normalized iodine concentration.

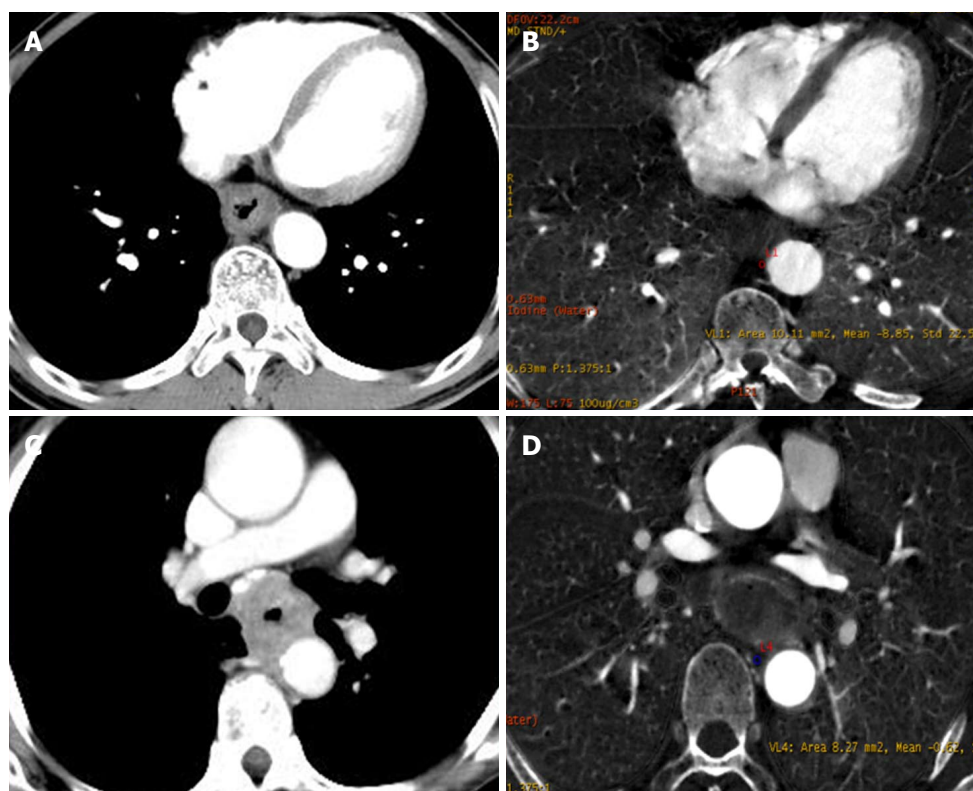
[(2.52 ± 1.39) mSv vs (1.63 ± 0.76) mSv vs (1.73 ± 0.44) mSv] among the groups ( $P > 0.05$ ). However, groups B and C received similar effective doses but lower iodine loads than group A [(300 vs 450) mgI/kg].

## DISCUSSION

Reducing the radiation dose while maintaining image quality has become a key issue in CT research<sup>[16]</sup>. Reasonable adjustments of the ASIR-weighted value with the appropriate reduction in the scanning conditions are the main factors for low-dose scanning<sup>[17]</sup>. As a type of automatic dynamic real-time radiation dose control technology, GSI appropriately changes the tube to compensate for the loss of image contrast by adjusting the field of view, rotation speed and detector width<sup>[18,19]</sup>. Our study shows that the CNRs of groups B and C were superior to the CNR of group A. Thus, GSI assist combined with ASIR achieved equal or higher image quality than conventional scanning. Low-dose scanning brings benefits to patients with esophageal cancer<sup>[20]</sup>. Radiation from multiple follow-ups can be potentially harmful to patients who receive multiple radiation or chemotherapy treatments.

The esophageal wall is composed of a mucosal layer, a submucosal layer, a muscle layer and an outer membrane layer. The infiltration depth of ESCC determines the T stage. In fact, esophageal wall thickening observed on CT enhancement is largely dependent on the pathological classification. We found that T staging not only depends on the advantages of imaging techniques, but is also closely connected to the degree of esophageal lumen filling. In addition, esophageal wall shrinkage leads to incomplete lumen filling and makes it difficult to identify layers. In our study, the proportion of layered enhancement for the medullary T1/2 and T3 stages increased when we performed EICT in group C. However, no significant differences between the T1/2 and T3 stages in the ulcer and mushroom types were found. We argue that insufflation CT promotes the ability to identify lesions located on one side or around the lumen, but limitations for flat masses or local ulcers remain.

We reported that there were no significant differences between the T3 and T4 stages in all types of ESCC when only observing the morphological changes of the triangular area in front of the vertebral body. We inferred that the changes in the triangular area in medullary



**Figure 6** Typical cases showing differences between the T3 and T4 stages of esophageal squamous cell carcinoma. A: Image from a 62-year old patient with T3N0M0 obtained using EICT combined GSI assist shows a clear triangular area in front of the vertebral body. B: IC in the triangle area during the AP was quantified to be  $-8.85 \mu\text{g}/\text{cm}^3$ , and NIC during the AP was  $-0.05 \mu\text{g}/\text{cm}^3$ . C: An axial image of a 57-year old patient obtained by EICT combined with GSI assist during the AP shows that the adipose tissue of the triangular area disappeared with suspected tumor invasion. D: IC in the triangle area during the AP was quantified to be  $-0.62 \mu\text{g}/\text{cm}^3$ , and NIC during the AP was  $-0.01 \mu\text{g}/\text{cm}^3$ . EICT: Esophageal insufflation computed tomography; IC: Iodine concentration; NIC: Normalized iodine concentration; AP: Arterial phase.

ESCC were affected by the size of the adipose tissue and connections with adjacent structures. Less adipose tissue and a close correlation with the triangular area are sometimes observed as blurring. Combined analysis of subjective observations and quantitative measurements revealed significant differences that could be used to discriminate T3 and T4 stage medullary ESCC in groups B and C. Hence, the better performance of the combined analysis in the triangular area was mainly attributed to the ability of NIC to discern invasion during the AP.

Based on the above analysis, the ability to diagnose T1-T3 stages is consistent with previous research<sup>[9,13]</sup>. The accuracy and sensitivity in distinguishing the T4 stage were similar to those reported by Cerfolio *et al.*<sup>[13]</sup> and Konieczny *et al.*<sup>[10]</sup>, while the specificity was significantly higher than that reported by Konieczny *et al.*<sup>[10]</sup>. Our study included more T4 stage patients than that of Konieczny *et al.*<sup>[10]</sup>, and, thus, our results may be more reliable than previous reports. Therefore, our findings demonstrate the relatively stable ability of our proposed technique to discriminate peripheral invasion, such as invasion of the trachea, aorta, muscle and pericardium.

There are several limitations of our study. First, the small sample size of T4 stage patients likely impacted the comparison of the diagnostic value in this preliminary study. Second, incomplete lumen filling associated with uncertain lesions restricts tumor localization. Third, nose

or mouth leaks were unavoidable when patients held their breath for a long period of time. Fourth, there is still a certain false-positive rate when analyzing the triangular area in front of the vertebral body. Furthermore, the EICT process is influenced by the patient's age and tolerance coordination. Lastly, other modalities, especially functional MRI, perform better in displaying the esophagus layer and distant metastases. Comparisons of EICT with EUS, MRI and PET-CT will be performed in the future. As mentioned above, fewer adipose tissue and close adjacent structural connections are easily misdiagnosed. The future direction of our research will focus on local expansion by lumen filling.

In conclusion, GSI optimizes the image contrast, maintains the radiation dose and reduces the contrast medium injection dose. EICT combined with GSI assist promotes differential diagnosis between T1/2 and T3 stage ESCC. The ability to differentially diagnose the T3 and T4 stages in medullary ESCC can be improved by quantitatively and qualitatively analyzing adipose tissue in front of the vertebral body.

## ARTICLE HIGHLIGHTS

### Research background

Conventional computed tomography (CT) has limitations for esophageal cancer staging or restaging after treatment. Diagnoses of the T1 and T2 stages exhibit



low accuracy due to difficulty visualizing the esophageal mucosa. The optimal monochromatic energy level clearly displays esophageal lesions and the surrounding adipose infiltration by effectively improving the image quality and resolution.

### Research motivation

Radiation from multiple follow-ups can be potentially harmful to patients who receive multiple radiation or chemotherapy treatments. Low-dose scanning brings benefits to patients with esophageal cancer. GSI combined with ASIR achieved image quality equal to or greater than that of conventional scanning.

### Research objectives

We aimed to evaluate the T stage of esophageal cancer using low-dose spectral insufflation CT, and we discuss the accuracy of this technique for preoperatively diagnosing the T stage.

### Research methods

One hundred and twenty patients with esophageal cancer were divided into three groups that included 45 patients (group A underwent conventional 120 kVp CT with 450 mgI/kg contrast medium injection), 40 patients (group B underwent GSI assist and 300 mgI/kg contrast medium injection) and 35 patients (group C underwent insufflation CT combined GSI assist and 300 mgI/kg contrast medium injection). Specific imaging features were observed, and the contrast-to-noise ratio of lesion-to-mediastinal adipose tissue was calculated for qualitative and quantitative T stage evaluation. The radiation dose was measured in each group.

### Research results

When performed with insufflation CT combined with GSI assist technology, the ability to present layered enhancement was significantly different for the identification of T1/2 and T3 stage medullary esophageal cancer. Combined analyses of the morphological features and normalized iodine concentration during the arterial phase in the triangular area in front of the vertebral body highlighted a significant difference in discriminating T3 and T4 stage medullary esophageal cancer.

### Research conclusions

EUS can be clinically used to determine the infiltration of esophageal cancer and the possibility of surgical resection. However, the detection range is limited to centimeters from the center of the ultrasonic probe without interference or severe stenosis. Currently, CT and PET/CT are common methods used to evaluate the T stage before esophageal cancer treatment. Hence, we aimed to evaluate the T stage of esophageal squamous cell carcinoma using low-dose spectral insufflation CT, and we discuss the accuracy of this technique for preoperatively diagnosing the T stage. We propose the new idea that the T stage for esophageal cancer can be assessed quantitatively and qualitatively methods using low-dose spectral CT scanning. We found that insufflation CT combined GSI assist technology allows a differential diagnosis between the T1/2 and T3 stages. The ability to differentially diagnose the T3 and T4 stages in medullary esophageal cancer can be improved by analyzing the adipose tissue in front of the vertebral body.

### Research perspectives

Nose or mouth leaks are unavoidable when patients hold their breath for a long period of time. Furthermore, the process of insufflation CT is influenced by the patient's age and ability to tolerate the procedures. The future direction of our research will focus on local expansion by lumen filling.

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## Novel methionyl-tRNA synthetase gene variants/phenotypes in interstitial lung and liver disease: A case report and review of literature

Kuerbanjiang Abuduxikuer, Jia-Yan Feng, Yi Lu, Xin-Bao Xie, Lian Chen, Jian-She Wang

Kuerbanjiang Abuduxikuer, Yi Lu, Xin-Bao Xie, Jian-She Wang, Department of Hepatology, Children's Hospital of Fudan University, Shanghai 201102, China

Jia-Yan Feng, Lian Chen, Department of Pathology, Children's Hospital of Fudan University, Shanghai 201102, China

Jian-She Wang, Department of Pediatrics, Jinshan Hospital of Fudan University, Shanghai 201508, China

ORCID number: Kuerbanjiang Abuduxikuer (0000-0003-0298-3269); Jia-Yan Feng (0000-0002-6651-4675); Yi Lu (0000-0002-3311-4501); Xin-Bao Xie (0000-0002-3692-7356); Lian Chen (0000-0002-0545-2108); Jian-She Wang (0000-0003-0823-586X).

**Author contributions:** Wang JS designed the report and approved the final submission; Abuduxikuer K collected data, analyzed relevant information, and wrote the manuscript; Wang JS, Lu Y, Xie XB, and Abuduxikuer K clinically managed the patient; Feng JY, Chen L analyzed liver biopsy samples.

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**Correspondence to:** Jian-She Wang, MD, PhD, Professor, Department of Hepatology, Children's Hospital of Fudan University, 399 Wanyuan Road, Shanghai 201102, China. [jshwang@shmu.edu.cn](mailto:jshwang@shmu.edu.cn)  
Telephone: +86-21-64931171  
Fax: +86-21-64931171

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### Abstract

Interstitial lung and liver disease (ILLD) is caused by biallelic mutations in the methionyl-tRNA synthetase (*MARS*) gene. To date, no genetic changes other than missense variants were reported in the literature. Here, we report a five-month old female infant with typical ILLD (failure to thrive, developmental delay, jaundice, diffuse interstitial lung disease, hepatomegaly with severe steatosis, anemia, and thrombocytosis) showing novel phenotypes such as kidney stones, acetabular dysplasia, prolonged fever, and extreme leukocytosis. Whole exome sequencing revealed a novel truncating variant (c.2158C>T/p.Gln720Stop) together with a novel tri-nucleotide insertion (c.893\_894insTCG that caused the insertion of an arginine at amino acid position 299) in the *MARS* gene.

**Key words:** Methionyl-tRNA synthetase; Infant; Kidney stone; Hip dysplasia; Leukocytosis; Interstitial lung and

liver disease; Methionyl-tRNA synthetase gene

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**Core tip:** Previously reported cases of interstitial lung and liver disease (ILLD) were associated with biallelic missense mutations in the methionyl-tRNA synthetase (*MARS*) gene. Here, we report a Chinese infant with typical ILLD (failure to thrive, developmental delay, interstitial lung disease, cholestasis, hepatomegaly, steatosis, anemia, and thrombocytosis) with novel phenotypes, such as kidney stones, acetabular dysplasia, prolonged fever, and extreme leukocytosis. Whole exome sequencing revealed a novel truncating variant (c.2158C>T/p.Gln720Stop), and a novel trinucleotide insertion (c.893\_894insTCG) in the *MARS* gene. Despite the resolution of cholestasis, this patient died of respiratory failure at the age of 11 mo.

Abuduxikuer K, Feng JY, Lu Y, Xie XB, Chen L, Wang JS. Novel methionyl-tRNA synthetase gene variants/phenotypes in interstitial lung and liver disease: A case report and review of literature. *World J Gastroenterol* 2018; 24(36): 4208-4216 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i36/4208.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i36.4208>

## INTRODUCTION

The methionyl-tRNA synthetase (*MARS*) gene encodes cytoplasmic methionyl-tRNA synthetase (MetRS) responsible for catalyzing the ligation of methionine to tRNA<sup>[1]</sup>. MetRS belongs to a family of aminoacyl-tRNA synthetases that play critical roles in protein biosynthesis by charging tRNAs with their cognate amino acids<sup>[2]</sup>. Interstitial lung and liver disease (ILLD) (OMIM#615486) is caused by homozygous or compound heterozygous mutations in the *MARS* gene (156560) on chromosome 12q13<sup>[3-5]</sup>. Heterozygous *MARS* mutations have been reported to be associated with autosomal dominant Charcot-Marie-Tooth disease (CMT)<sup>[6-9]</sup>. The same *MARS* mutation may cause both ILLD and CMT<sup>[10]</sup>. *MARS* is also a candidate gene for hereditary spastic paraplegias (HSPs), a neuro-degenerative motor neuron disorder<sup>[11]</sup>. To date, no genetic changes other than missense variants have been reported in the literature. Here, we report a Chinese infant with lethal ILLD showing novel phenotypes such as kidney stones, acetabular dysplasia, prolonged fever, and extreme leukocytosis. Whole exome sequencing revealed a novel truncating variant together with a novel tri-nucleotide insertion in the *MARS* gene.

## CASE REPORT

A five-month old female infant was presented with a

failure to thrive, developmental delay, jaundice, and dark urine. She was born full-term with a normal birth weight (3100 g) after an uncomplicated first pregnancy and vaginal delivery. Weight gain and developmental milestones were normal until three months of age (weighted 6000 g), when she failed to thrive with a body weight of 5700 g at the age of 5 mo without the ability of rolling over.

At in-patient admission, this patient was 5.2 mo old with a body weight of 5500 g (2<sup>nd</sup> percentile by WHO standards), length of 55 cm (lower than the 1<sup>st</sup> percentile), and head circumference of 39 cm (2<sup>nd</sup> percentile). This infant had prolonged low-grade fever, pulmonary effusion, diffuse interstitial lung disease, significant leukocytosis, high procalcitonin (PCT)/CRP levels, and required nasal oxygen therapy. Serial chest X-rays showed some improvement in pulmonary effusion, but no improvement in interstitial lung involvement (Figure 1A). After serial antibiotic treatments (ceftriaxone, cefoperazone + sulbactam, meropenem, norvancomycin, and fluconazole), body temperature was normalized, oxygen therapy was no longer needed, and leukocytosis improved, however the interstitial lung disease stayed the same. After treatment with ursodeoxycholic acid and fat-soluble vitamins, cholestasis improved significantly (Table 1).

The patient was discharged with normal oxygen saturation on room air without apparent respiratory distress or cough. A liver function test and complete blood count were normal at a 9.5 mo follow-up. However, the infant was admitted to a provincial level pediatric intensive care unit for acute respiratory distress at 11 mo of age and received mechanical ventilation. Despite treatment, she died of respiratory failure and hypoxic encephalopathy.

A genetic cause was suspected due to multiple system involvement, although a liver panel consisting of 41 genes (Table 2) related to liver diseases came back negative. Lysosomal storage disease was considered, but an enzyme panel for the screening of common lysosomal storage diseases was normal, as was the urine acidoglycoprotein level. This patient was enrolled for the undiagnosed disease patient program in our hospital, and whole exome sequencing was ordered. Compound heterozygous *MARS* gene variants, c.2158C>T/p.Gln720Stop and c.893\_894insTCG/p.Arg299dup, were detected. Presence of these mutations was confirmed with Sanger sequencing, and parental origins were ascertained. Both variants were not reported in the dbSNP137 (<http://www.ncbi.nlm.nih.gov/snp/>), 1000 Genome Database (<http://www.1000genomes.org/>), and Exome Variant Server (<http://evs.gs.washington.edu/EVS/>). The c.2158C>T mutation was inherited from the healthy mother, which caused the change of a glutamine amino acid at position 720 to a stop codon, which was predicted to be disease-causing by MutationTaster (<http://www.mutationtaster.org/>). The tri-nucleotide insertion (c.893\_894insTCG)



**Table 1** Changes in complete blood count, procaltitonin, serum biochemistry, and blood coagulation profiles

| Age (mo) ( <sup>1</sup> in-patient admission; <sup>2</sup> discharge to out-patient follow-up) |   | 5.1   | 5.6 <sup>1</sup> | 6     | 6.2   | 6.5   | 6.8   | 7     | 7.2 <sup>2</sup> | 9.5   |
|--|---|-------|------------------|-------|-------|-------|-------|-------|------------------|-------|
| Complete blood count (reference range)   | White blood cell ( $4-10 \times 10^9/L$ )           | 16.9  | 21.1             | 71.7  | 26.4  | 33.7  | 45.8  | 30.3  | 24.3             | 14.3  |
|  | Neutrophil (20%-50%)                                | 58.1  | 39.8             | 58.0  | 63.1  | 62.0  | 63.0  | 62.7  | 64.4             | 38.9  |
|  | Lymphocyte (45%-75%)                                | 36.2  | 53.1             | 31.1  | 28.7  | 28.9  | 15.0  | 29.5  | 27.8             | 51.4  |
|  | Abnormal lymphocytes (0%)                           | NA    | 0.0              | 0.0   | NA    | 0.0   | 17.0  | 0.0   | 0.0              | NA    |
|  | Platelet count ( $100-300 \times 10^9/L$ )          | 764.0 | 513.0            | 993.0 | 464   | 387.0 | 494.0 | 279.0 | 397              | 386.0 |
|  | Hemoglobin (110-160 g/L)                            | 78.0  | 85.2             | 78.2  | 60.1  | 64.0  | 65.2  | 90.0  | 88.0             | 122.0 |
|  | Red blood cell count ( $4.0-5.5 \times 10^{12}/L$ ) | 3.5   | 3.1              | 2.8   | 2.0   | 2.0   | 2.2   | 2.9   | 2.9              | 4.3   |
|  | Reticulocyte (0.5%-1.5%)                            | NA    | 2.9              | 6.7   | NA    | 6.3   | 7.8   | 3.3   | 6.8              | 1.0   |
| Procaltitonin (< 0.05 ng/mL)   | C-reactive protein (< 8 mg/L)                       | 1.0   | 8.0              | 90.0  | 32.0  | 43.0  | 37.0  | 45.0  | 8.0              | 8.0   |
|  | Albumin (35-55 g/L)                                 | 29.0  | 34.6             | 27.3  | 30.8  | 32.3  | 28.7  | 38.5  | 39.1             | 43.0  |
|  | Alanine aminotransferase (0-40 IU/L)                | 41.0  | 45.0             | 17.0  | 13.0  | 4.0   | 50.0  | 49.0  | 38.0             | 29.0  |
|  | Aspartate aminotransferase (0-40 IU/L)              | 100.0 | 104.0            | 46.0  | 37.0  | 66.0  | 98.0  | 70.0  | 62.0             | 41.0  |
|  | Total bilirubin (5.1-17.1 $\mu$ mol/L)              | 68.0  | 120.4            | 133.0 | 132.9 | 126.8 | 110.6 | 90.1  | 42.9             | 8.1   |
|  | Direct bilirubin (0-6 $\mu$ mol/L)                  | 53.0  | 76.9             | 93.7  | 96.1  | 86.6  | 70.4  | 61.8  | 29.8             | 4.4   |
|  | $\gamma$ -glutamyl transferase (7-50 IU/L)          | 73.0  | 61.0             | 76.0  | 58.0  | 54.0  | 57.0  | 107.0 | 230.0            | 122.0 |
|  | Total bile acid (0-10 $\mu$ mol/L)                  | NA    | 182.8            | 123.3 | 152.4 | 137.2 | 157.4 | 311.7 | 282.3            | 34.6  |
| Serum biochemistry (reference range)   | Alkaline phosphatase (42-383 IU/L)                  | 307.0 | 137.0            | 149.0 | 119.0 | 122.0 | 148.0 | 178.0 | 214.0            | 378.0 |
|  | Blood glucose (3.9-5.8 mmol/L)                      | NA    | 1.2              | 1.6   | 8.4   | 1.1   | NA    | NA    | 3.6              | NA    |
|  | Lactic acid (0-2 mmol/L)                            | NA    | 3.9              | NA    | 3.6   | 3.6   | NA    | NA    | NA               | NA    |
|  | Ammonia (10-47 $\mu$ mol/L)                         | NA    | 88.0             | NA    | NA    | NA    | NA    | NA    | 55.0             | NA    |
|  | Total cholesterol (3.1-5.2 mmol/L)                  | 3.1   | 2.0              | NA    | 2.3   | 2.5   | NA    | 2.8   | 4.4              | 3.1   |
|  | LDL-cholesterol (1.30-3.90 mmol/L)                  | NA    | NA               | NA    | 1.0   | NA    | NA    | NA    | NA               | NA    |
|  | HDL-cholesterol (0.91-2.05 mmol/L)                  | NA    | NA               | NA    | 0.3   | NA    | NA    | NA    | NA               | NA    |
|  | Triglyceride (0.56-1.70 mmol/L)                     | NA    | 2.0              | NA    | 2.7   | 2.1   | NA    | 2.1   | 1.8              | 1.5   |
| Blood coagulation profiles (reference range)   | Activated partial thromboplastin time (28.0-44.5 s) | NA    | 48.1             | NA    | 57.5  | 56.4  | 53.9  | 47.7  | 42.3             | 43.8  |
|  | D-dimer (0-0.3 mg/L)                                | NA    | 0.94             | NA    | 2.06  | 1.15  | 0.97  | 0.7   | 0.51             | NA    |
|  | Fibrinogen (2-4 g/L)                                | NA    | 1.45             | NA    | 1.82  | 2.29  | 2.54  | 3.03  | 3.46             | 3.44  |
|  | Fibrinogen degradation products (0-5 $\mu$ g/mL)    | NA    | 1.31             | NA    | 5.22  | 2.35  | 2.78  | 1.47  | 1.16             | NA    |
|  | Thrombin time (14-21 s)                             | NA    | 20.4             | NA    | 19.1  | 19.9  | 19.9  | 15.8  | 18.4             | 15.2  |
|  | International normalized ratio (0.8-1.2)            | NA    | NA               | NA    | 1.29  | 1.26  | 1.35  | 1.3   | 1.03             | 0.99  |
|  | Prothrombin time (12.0-14.8 s)                      | NA    | NA               | NA    | 16    | 15.7  | 16.5  | 16.1  | 13.5             | 13.1  |
|  | Prothrombin time activity (80%-100%)                | NA    | NA               | NA    | 67    | 69    | 63    | 66    | 95               | 103   |

NA: Not available.

inherited from her healthy father caused the insertion of a single amino acid (arginine) at position 299, which was predicted to be disease-causing by MutationTaster (Figure 2A). The detailed genetic testing results and secondary findings are provided in Table 2.

Liver biopsy results showed severe steatosis of hepatic cells with ballooning, lobular disarray, and cholestasis. Mild changes, such as fibrosis, lymphocyte infiltration, and bile duct proliferation, were seen within the portal region. Hepatic iron deposition was seen after iron staining, but copper staining was negative (Figure 1C). Sinusoids and Kupffer cells seemed normal. Immunohistochemical staining for hepatitis B surface antigen, core antigen, Epstein-Barr virus, and langerin cells were negative. Immunohistochemical staining for cholestasis-related proteins, such as BSEP, MDR3, MRP2, TJP2, and MYO5B, were all normal. After genetic diagnosis, we used a rabbit anti-MARS monoclonal antibody (purchased from <http://www.abcam.cn>, product code: ab180497) to perform immunohistochemical staining on paraffin-embedded liver biopsy samples. When compared to a normal liver sample (donated for liver transplantation), coarsely granular pigments within the cytoplasm were seen in

the index patient sample.

Ultrasound examination revealed marked hepatomegaly (liver 4 cm below the right costal margin, and 5 cm below the xiphoid process) and reduced hepatic echogenicity. Hyper-echoic lesions consistent with stone formation were seen on both kidneys. Abdominal computed tomography scans showed hepatic steatosis and hyper-echoic lesions suggestive of kidney stones in the left kidney but not in the right kidney (Figure 1B). X-ray imaging of the skull was normal, as were the long bones of both arms and legs. X-ray imaging also picked up abnormally shallow hip sockets on both sides, which is suggestive of acetabular dysplasia or congenital hip dysplasia (Figure 1B). Other diagnostic evaluations are provided in Table 3.

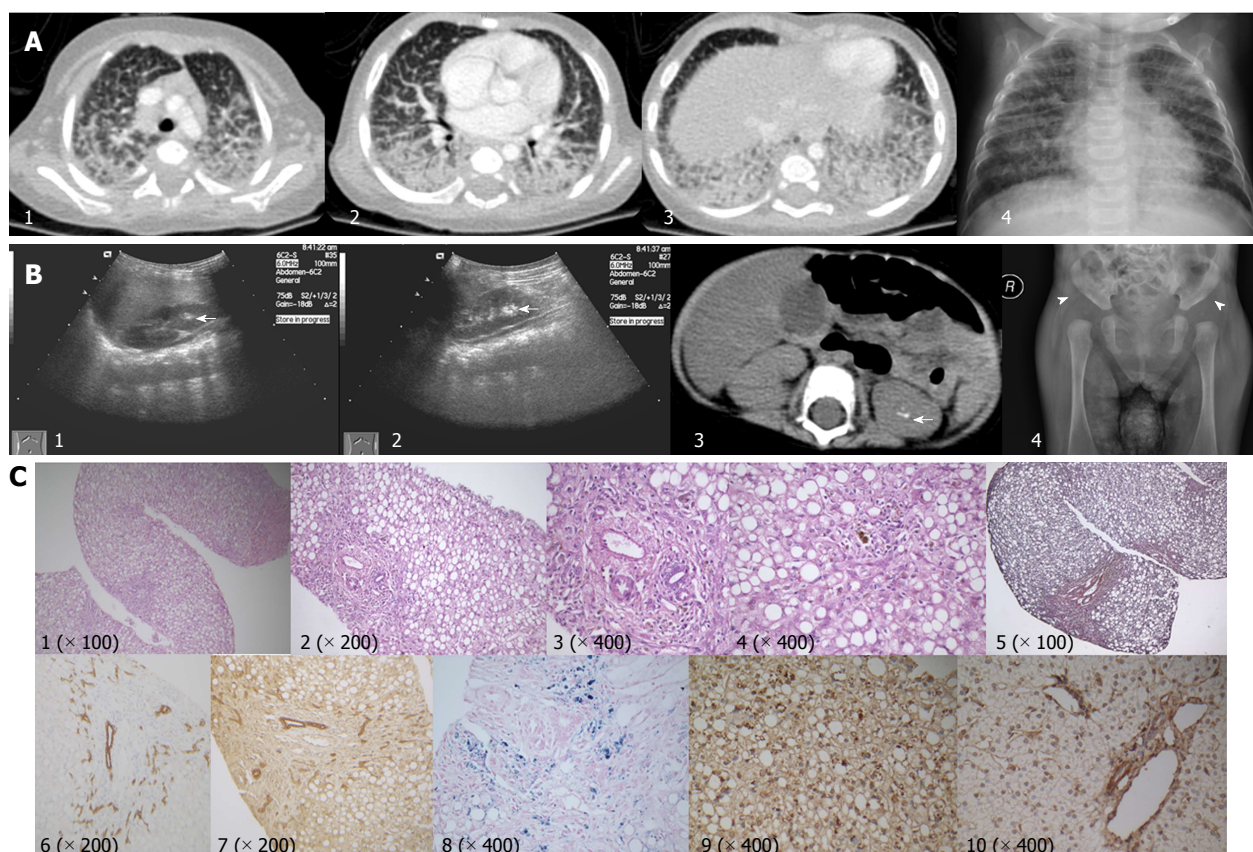
## DISCUSSION

MetRS is one of 20 ubiquitously expressed enzymes essential for protein biosynthesis, and covalently links methionine with its cognate tRNA. Since initial reports of *MARS* gene mutations causing ILLD<sup>[3]</sup> and CMT<sup>[6]</sup> in 2013, a total of 34 cases of ILLD<sup>[4,5,10]</sup> and eight cases of CMT<sup>[7-10]</sup> have been reported.

Table 2 Genetic testing results

| Genetic Tests            | Gene                          | Transcript ID          | Associated conditions (Inheritance patterns) in OMIM   | Variant         | Amino-acid change | Hom/Het | Parental origin | Mutation taster | Prediction of pathogenicity | Polyphen2         |
|--------------------------|-------------------------------|------------------------|--|-----------------|-------------------|---------|-----------------|-----------------|-----------------------------|-------------------|
| Liver Panel <sup>1</sup> | <i>ATP8B1</i>                 | NM_005603              | Cholestasis, benign recurrent, intrahepatic (AR); cholestasis, intrahepatic, of pregnancy, 1 (AD); cholestasis, progressive familial intrahepatic 1 (AR)   | c.234C>G        | p.His78Gln        | Het     | NA              | Polymorphism    | Tolerated                   | Benign            |
|                          |                               |                        |  | c.1729A>G       | p.Ile577Val       | Het     | NA              | Polymorphism    | Tolerated                   | Possibly damaging |
|                          |                               |                        |  | c.2021T>C       | p. Met674Thr      | Het     | NA              | Polymorphism    | Tolerated                   | Benign            |
| Whole exome sequencing   | <i>MARS</i>                   | NM_004990              | Charcot-Marie-Tooth disease, axonal, type 2U (AD); Interstitial lung and liver disease (AR)  | c.3477C>T       | Synonymous        | Het     | NA              | Polymorphism    | Tolerated                   | Benign            |
|                          |                               |                        |  | c.3744C>A       | Synonymous        | Het     | NA              | Polymorphism    | Tolerated                   | NA                |
|                          |                               |                        |  | c.2158C>T       | p.Gln720Stop      | Het     | Maternal        | Disease causing | NA                          | NA                |
|                          | <i>ATP8B1</i>                 | NM_005603              | Cholestasis, benign recurrent, intrahepatic (AR); cholestasis, intrahepatic, of pregnancy, 1 (AD); cholestasis, progressive familial intrahepatic 1 (AR)   | c.893_894insTCG | p.Arg299dup       | Het     | Paternal        | Disease causing | NA                          | Deleterious       |
|                          |                               |                        |  | c.2021T>C       | p. Met674Thr      | Het     | Paternal        | polymorphism    | Tolerated                   | Benign            |
|                          |                               |                        |  | c.1163+5G>A     | -                 | Het     | Maternal        | Disease causing | NA                          | NA                |
|                          | <i>CPT1A</i><br><i>LRPPRC</i> | NM_001876<br>NM_133259 | CPT deficiency, hepatic, type 1A (AR)<br>Leigh syndrome, French-Canadian type (AR)   | c.2965C>T       | p.Arg989Cys       | Het     | Maternal        | Disease causing | Damaging                    | Probably damaging |
|                          |                               |                        |  | c.5841G>A       | p.Trp1947Stop     | Het     | Maternal        | Disease causing | NA                          | NA                |
|                          |                               |                        |  | c.241C>T        | p.Arg81Cys        | Het     | Maternal        | Disease causing | Damaging                    | Benign            |
|                          | <i>FLG</i>                    | NM_002106              | Ichthyosis vulgaris (AD); (Dermatitis, atopic, susceptibility to, 2)   | c.794G>A        | p.Arg265His       | Het     | Maternal        | Disease causing | Damaging                    | Probably damaging |
|                          | <i>G6PD</i>                   | NM_00104251            | Hemolytic anemia, G6PD deficient (favism) (XLD); (Resistance to malaria due to G6PD deficiency)  |                 |                   |         |                 |                 |                             |                   |
|                          | <i>POMGNT1</i>                | NM_017739              | Muscular dystrophy-dystroglycanopathy (congenital with brain and eye anomalies), type A, 3 (AR); Muscular dystrophy-dystroglycanopathy (congenital with mental retardation), type B, 3 (AR); Muscular dystrophy-dystroglycanopathy (limb-girdle), type C, 3 (AR); Retinitis pigmentosa 76 (AR) |                 |                   |         |                 |                 |                             |                   |
|                          | <i>SERPINC1</i>               | NM_000488              | Thrombophilia due to antithrombin III deficiency (AD/AR)   | c.719A>G        | p.Asn240Ser       | Het     | Maternal        | Polymorphism    | Tolerated                   | Benign            |
|                          | <i>TG</i>                     | NM_003235              | Thyroid dysmorphogenesis 3 (AR); (autoimmune thyroid disease, susceptibility to, 3)  | c.5791A>G       | p.Ile1931Val      | Het     | Paternal        | Polymorphism    | Tolerated                   | Benign            |
|                          | <i>USH2A</i>                  | NM_206933              | Retinitis pigmentosa 39; Usher syndrome type 2A (AR)   | c.8559-2A>G     | -                 | Het     | Paternal        | Disease causing | NA                          | NA                |

<sup>1</sup>Genes included in liver panel: *ATP8B1*, *ABCB4*, *TJP2*, *BAAT*, *CLDN1*, *HSDB3B7*, *AKR1D1*, *CYP7B1*, *AMACR*, *CYP27A1*, *DHCR7*, *JAG1*, *NOTCH2*, *SLC25A13*, *DGUK*, *MPV17*, *FAH*, *ABCC2*, *UGT1A1*, *NPC1*, *NPC2*, *GALT*, *GALE*, *ALDOA*, *ALDOB*, *KRT18*, *KRT8*, *CIRH1A*, *CFTR*, *GDFM1*, *EARS2*, *HSB17B4*, *LIPA*, *PEX1*, *PEX5*, *POU1F1*, *HESX1*, *SERPINA1*, *VIPAS39*, and *VPS33B*. NA: Not available.



**Figure 1** Imaging and histopathological features. A: Contrast enhanced pulmonary CT scan (1-3), and chest X-ray (4) showing pulmonary effusion with marked interstitial lung involvement; B: Hyper-echoic lesions consistent with stone formation on ultrasonography (arrows; 1, right kidney; 2, left kidney) and non-contrast abdominal computed tomography scan (arrow, 3). Acetabular dysplasia (4, arrowhead showing abnormally shallow hip socket); C: Liver biopsy (all originally magnified principal images): severe steatosis of hepatic cells with ballooning, lobular disarray, and cholestasis (1-4), mild fibrosis (5), mild lymphocyte infiltration (4), bile duct proliferation (6 CK-7, 7 CK-19), and hepatic iron deposition (8). *MARS* immunohistochemistry staining, coarsely granular pigments within the cytoplasm in the index patient (9), but not in samples of a healthy control (10). *MARS*: Methionyl-tRNA synthetase gene.

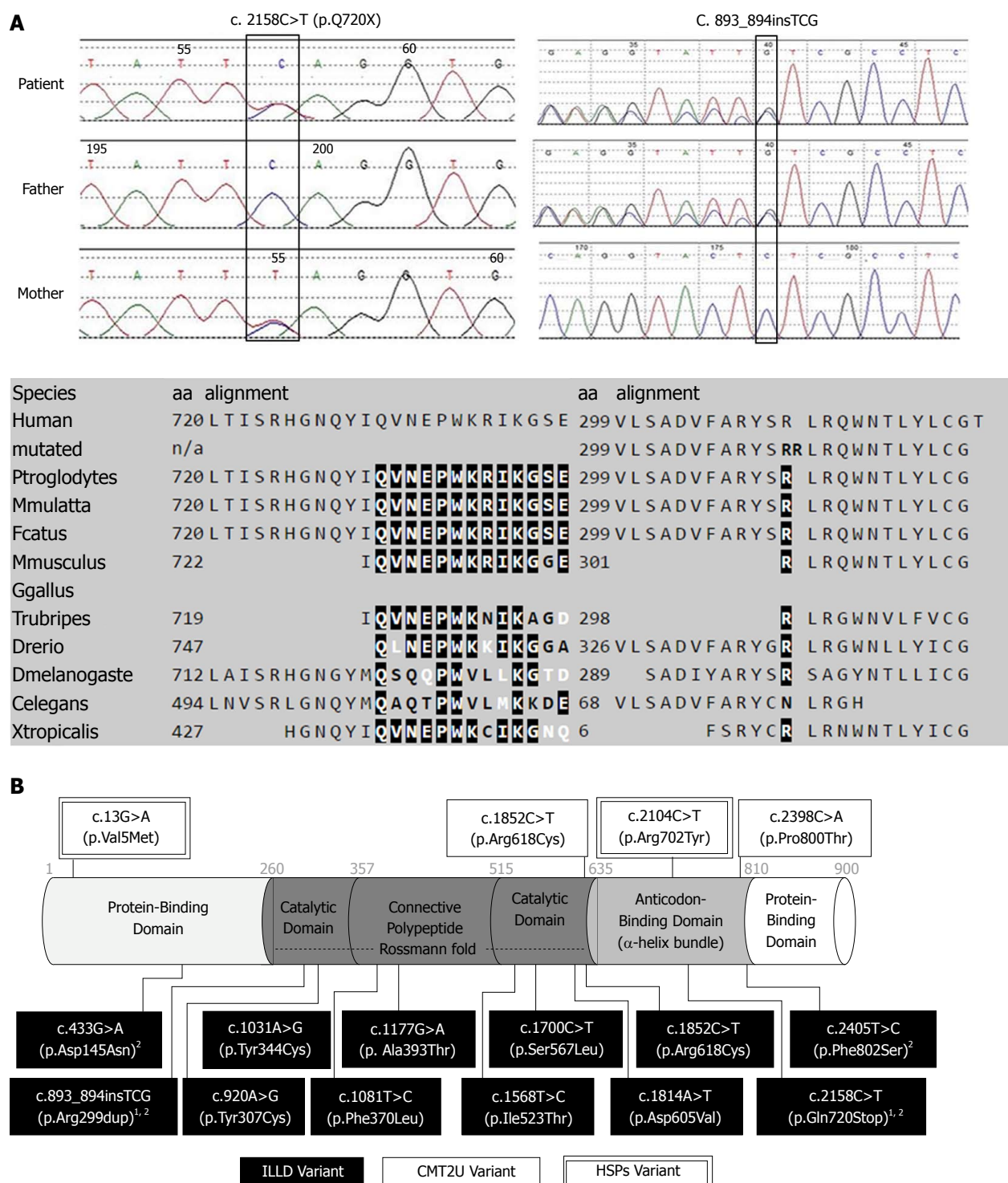
Similar to previous reports, the patient in our case showed a failure to thrive, developmental delay, interstitial lung disease, liver involvement (hepatomegaly, cholestasis, hepatic steatosis, fibrosis, and iron deposition), anemia, and thrombocytosis. An active proliferation of bone marrow cells has been reported by Sun *et al.*<sup>[5]</sup>. Our patient had marked leukocytosis (white blood cell count up to  $71.7 \times 10^9/L$ ), and a bone marrow biopsy showed extreme proliferation of bone marrow cells with few hemophagocytic cells. MetRS is also a component of a cytoplasmic multi-aminoacyl-tRNA synthetase complex with multiple roles in immune response, inflammation, and tumorigenesis<sup>[12,13]</sup>. Prolonged low-grade fever, leukocytosis, thrombocytosis, and elevated c-reactive protein in this patient responded to intensive antibiotic treatment, and could be viewed as an exaggerated inflammatory or immune response to infection. Unlike previous reports of an arrest in red blood cell maturity<sup>[3,5]</sup>, a bone marrow biopsy from this patient showed marked proliferation of normal erythrocyte precursors.

While aminoaciduria has been reported<sup>[3]</sup>, kidney stones have never been reported to be associated with a *MARS* mutation. No evidence of urinary tract infection,

proteinuria, or organic aciduria was found in our case, and serum electrolytes with urea and creatinine were essentially normal. An evaluation of urinary citrate, calcium, and 24 h urine output in future ILLD cases might be necessary in order to rule out factors that promote renal stone formation<sup>[14]</sup>. Mutations in genes encoding mitochondrial seryl-tRNA synthetases have been reported to cause renal damage<sup>[15,16]</sup>, but no association of cytoplasmic aminoacyl-tRNA synthetases, including *MARS*, have been reported. Since previously reported mutations were all non-synonymous in nature, severe mutations (such as a truncation or single amino acid insertion as in our case) may have caused some renal impairment leading to stone formation.

No skeletal abnormality has been reported, with the exception of two ILLD cases with delayed bone age<sup>[5]</sup>. Our case had marked acetabular dysplasia consistent with developmental hip dysplasia. Other than being female, this infant did not have other risk factors<sup>[17]</sup>, such as breach presentation upon delivery, local infection, or trauma. Whole exome sequencing did not reveal abnormalities in previously reported susceptible genes such as *GDF5*, *TBX4*, *ASPN*, *IL-6*, *TGF- $\beta$ 1*, and *PAPPA2*<sup>[18]</sup>. Hip dysplasia is associated with CMT<sup>[19]</sup>,





**Figure 2** Genetic testing results, protein features, and distribution of reported variants within the methionyl-tRNA synthetase protein. A: Sanger sequencing confirmation of the index case and parents, both variants affect highly conserved amino acid residues of the MetRS protein; B: Illustration of MetRS protein domains, location of amino acid changes of the reported variants so far. <sup>1</sup> Variants from our report; <sup>2</sup> Variants from Chinese ILLD cases. MetRS: Methionyl-tRNA synthetase; ILLD: Interstitial lung and liver disease.

and the rate of hip dysplasia among children with CMT ranges from 6% to 8.1%<sup>[20]</sup>. Novarino *et al.*<sup>[11]</sup> reported four cases of HSPs with compound heterozygous variants of the *MARS* gene in a family with infantile onset delayed motor milestones and disabilities upon crawling/walking. Two cases had bilateral Achilles

contracture, one had scoliosis, but none had hip-joint abnormalities. A recent report of an ILLD case<sup>[10]</sup> with a p.Arg618Cys variant was also associated with CMT in a previous report<sup>[6]</sup>, indicating ILLD and CMT may share a similar disease-causing mechanism. All reported cases of CMT, ILLD, and HSPs associated with the *MARS* gene



**Table 3** Diagnostic evaluation of the patient with a methionyl-tRNA synthetase mutation

| Etiological assessment                         | Investigations performed (normal unless otherwise indicated)   |
|--|--|
| Infections                                     | Serum procaltitonin levels (significantly elevated, Table 1);<br>Serology for Hepatitis B, C, HIV, syphilis, EBV, CMV, HSV, toxoplasmin, and rubella virus;<br>PCR for CMV; beta-d-glucan assay; galactomannan assay; T-Spot.TB test;<br>Cerebrospinal fluid analysis for white blood cell count, protein, and glucose level;<br>Complete blood count: anemia, elevated WBC and C-reactive protein (Table 1);<br>Culture for blood, urine, sputum, alveolar lavage fluid, and cerebrospinal fluid;<br>Sputum and alveolar lavage fluid for mycoplasma/chlamydia DNA detection;<br>Sputum and alveolar lavage fluid for detection of respiratory syncytial virus, adenovirus, influenza virus, and para-influenza virus antigens;<br>Alveolar lavage fluid smear for fungus detection   |
| Radiology, endoscopy, and histopathology       | Multiple chest X-rays and a contrast-enhanced computed tomography scan of the lung (alveolar effusions with severe interstitial lung disease) (Figure 1);<br>Abdominal ultrasonography and CT scan (hepatomegaly, liver steatosis, kidney stones) (Figure 1);<br>Bronchoscopy (chronic inflammatory changes in bronchiolar mucosa);<br>X-ray imaging of the skull; CT scan of adrenal gland;<br>X-ray imaging of long bones: (abnormally shallow hip socket that is suggestive of acetabular dysplasia or congenital hip dysplasia) (Figure 1);<br>Liver biopsy (severe steatosis of hepatic cells with ballooning, lobular disarrays; mild changes, such as cholestasis, fibrosis, lymphocyte infiltration, Iron deposition, and bile duct proliferation);<br>Bone marrow aspirate (extreme proliferation of bone marrow cells with few hemophagocytic cells); peripheral blood smear |
| Immunology                                     | Immunoglobulin levels (after IVIG therapy at local hospital): elevated IgG (20.2 g/L, normal range 3.7-8.3 g/L), IgM (1.47 g/L, normal range 0.33-1.25 g/L), and IgA (0.63 g/L, normal range 0.14-0.5) levels; normal IgE, complement 4, and complement 3 levels;<br>Neutrophil oxidative burst activity, and lymphocyte subpopulations;<br>Autoimmune antibodies  |
| Biochemical, metabolic and endocrine profiling | Glucose profiling (hypoglycemia); slightly elevated serum lactate (Table 1);<br>Liver function test: cholestasis, hypoalbuminemia, abnormal blood coagulation profiles (Table 1);<br>Creatine kinase, lactate dehydrogenase;<br>Serum amino acids (proline 1803 $\mu\text{mol/L}$ , normal range: 165-700 $\mu\text{mol/L}$ ; threonine 171 $\mu\text{mol/L}$ , normal range: 17-90 $\mu\text{mol/L}$ ) and acyl-carnitine profile; urine organic acids (including succinylacetone); Urine acidoglycoprotein (51.98 mg/mmol creatinine, normal range: 59.70-78.52 mg/mmol creatinine).<br>Low levels of total serum cholesterol, HDL and LDL cholesterol (Table 1).<br>Serum cortisol level; thyroid function test (total triiodothyronine 52.6 ng/dL, normal range: 70-220 ng/dL)<br>Ophthalmology, electrocardiology, and echocardiogram (patent foramen ovale, 2.6 mm)              |
| Genetic disorders                              | White blood cell lysosomal enzyme screening for GM1 gangliosidosis, GM2 gangliosidosis, Sandhoff disease, Krabbe leukodystrophy, Gaucher disease, Fabry disease, Pompe disease, metachromatic leukodystrophy, Nieman-Pick disease, neuronal ceroid lipofuscinoses (1 and 2), mucopolysaccharidosis (type I-VII, IX), muculipidosis (type II and III).<br>Liver panel including 41 genes known to cause liver diseases, and trio whole exome sequencing (Table 2).  |

had missense mutations. Our case had a truncating mutation and an insertion of a single amino acid. Severe mutations may have been responsible for the hip dysplasia, which could be an early manifestation of CMT in this patient.

The c.2158C>T/p.Gln720Stop, which was inherited from the mother, caused the glutamine amino acid change at position 720, leading to a stop codon at a well-conserved  $\alpha$ -helix bundle domain (anti-codon binding domain) of the methionyl-tRNA synthetase protein.

The tri-nucleotide insertion (c.893\_894insTCG) with paternal origin caused the insertion of a single amino acid (arginine) at position 299 in the Rossmann fold domain (catalysis center). Nine out of 12 ILLD variants reported so far affected an amino acid in the Rossmann fold domain (Figure 2B). Arg299 is adjacent to the active methionine-binding site of human MetRS, which is surrounded by the amino acid residues Arg12, Leu13, Pro14, Thr257, Gly259, Tyr260, Asn297, and His301<sup>[21]</sup>.

All eight mutations from European ILLD cases were

located in the Rossmann fold of the MARS protein. However, only one out of four mutations from Chinese cases carried mutations in the Rossmann fold domain, and the location of mutations among Chinese ILLD cases was significantly different from that of European ILLD cases (Fisher's exact = 0.018) (Figure 2B). Our case also suggested that severe mutations may lead to more organ/system involvement and severe outcomes.

*In vivo* yeast complementation assays were used to predict the effects of *MARS* variants, including 1852C>T/p.Arg618Cys<sup>[6]</sup>, c.920A>G/p.Tyr307Cys<sup>[10]</sup> and 1852C>T/p.Arg618Cys<sup>[10]</sup>. The *in vitro* aminoacylation assay with HEK293 cells was used to confirm the effects of c.1108T>C/p.Phe370Leu, and c.1568T>C/p.Ile523Thr *MARS* variants<sup>[3]</sup>. The effects of c.1031A>G/p.Tyr344Cys, c.1177G>A/p.Ala393Thr, c.1700C>T/p.Ser567Leu and c.1814A>T/p.Asp605Val were studied using the *in vitro* yeast aminoacylation assay<sup>[4]</sup>, and later by Comisso *et al.*<sup>[22]</sup> using the *E. Coli*-based aminoacylation assay. Further functional

studies are needed to confirm the effects of variants in our case, as well as variants reported by others (c.2398C>A/p.Pro800Thr<sup>[7]</sup>, c.433G>A/p.Asp145Asn and c.2405T>C/p.Phe802Ser<sup>[5]</sup>). Besides previously used methods, one may consider the use of animal models such as *Drosophila* and *C. elegans* to predict the pathogenicity of other aminoacyl-tRNA synthetase mutations<sup>[23]</sup>.

There is currently no cure for ILLD, and thus treatment is only supportive. Provided that *in vitro* enzyme activity may partly be restored by increasing methionine<sup>[22]</sup>, methionine supplementation could be considered in studies of animal models, or possibly even in humans. However, plasma levels of methionine and its toxic product homocysteine should be closely monitored.

In conclusion, truncation and insertion variants in the *MARS* gene may cause ILLD, and phenotypes of ILLD may also include kidney stones, acetabular dysplasia, prolonged fever, and extreme leukocytosis.

## ARTICLE HIGHLIGHTS

### Case characteristics

A five-month old female infant presented with failure to thrive, developmental delay, jaundice, and dark urine.

### Clinical diagnosis

Typical clinical findings and whole exome sequencing results led to a diagnosis of interstitial lung and liver disease (ILLD).

### Differential diagnosis

Genetic cause was suspected due to multiple system involvement, but a liver panel consisting of 41 genes related to liver diseases came back negative. Lysosomal storage disease was considered, but an enzyme panel for screening common lysosomal storage diseases was normal, as was the urine acidoglycoprotein level.

### Laboratory diagnosis

Laboratory findings were Cholestasis, anemia, abnormal blood coagulation profiled, thrombocytosis, and extreme leukocytosis. Whole exome sequencing revealed a novel truncating variant (c.2158C>T/p.Gln720Stop) and a novel tri-nucleotide insertion (c.893\_894insTCG) in the methionyl-tRNA synthetase (*MARS*) gene.

### Imaging diagnosis

X-ray, computed tomography scan, and ultrasound imaging revealed interstitial lung disease, hepatomegaly, kidney stones, and acetabular dysplasia.

### Pathological diagnosis

Liver biopsy results showed severe hepatic steatosis, hepatic cells ballooning, lobular disarray, cholestasis, iron deposition, and mild fibrosis/lymphocyte infiltration/bile duct proliferation within the portal region.

### Treatment

Ursodeoxycholic acid, fat-soluble vitamins, antibiotics, oxygen therapy, and supportive treatment.

### Related reports

Previous reports of ILLD were associated with biallelic missense mutations in the *MARS* gene. Phenotypes, such as kidney stones, acetabular dysplasia, prolonged fever, and extreme leukocytosis have never been reported to be

associated with ILLD.

### Term explanation

ILLD is interstitial lung and liver disease caused by homozygous or compound heterozygous mutations in the *MARS* gene. Typical findings in ILLD include failure to thrive, developmental delay, interstitial lung disease, liver involvement (hepatomegaly, cholestasis, hepatic steatosis, fibrosis, and iron deposition), anemia, and thrombocytosis.

### Experiences and lessons

Regardless of race or ethnicity, ILLD should be considered in all patients with chronic liver diseases showing progressive interstitial lung involvement. Severe mutations may lead to more organ/system involvement and severe outcomes.

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