Title: **acFibroMASH index for the diagnosis of fibrotic MASH and prediction of liver-related events: An international multicenter study**

Short Title: acFibrotic MASH index new insights

Authors:

Gong Feng[1,2#], Ferenc E Mózes[3#], Dong Ji[4], Sombat Treeprasertsuk[5], Takeshi Okanoue[6], Toshihide Shima[6], Huiqing Liang[7], Emmanuel Tsochatzis[8], Jinjun Chen[9], Jörn M. Schattenberg[10,11], Christian Labenz[10,11], Sanjiv Mahadeva[12], Wah Kheong Chan[12,] Xiaoling Chi[13], Adèle Delamarre[14], Victor de Lédinghen[14], Salvatore Petta[15], Elisabetta Bugianesi[16] , Hannes Hagström[17,18], Jérôme Boursier[19,20], José Luis Calleja[21], George Boon-Bee Goh[22], Rocio Gallego-Durán[23], Arun J. Sanyal[24], Jian-Gao Fan[25], Laurent Castéra[26], Michelle Lai[27], Stephen A. Harrison[28,29], Manuel Romero-Gomez[23], Seung Up Kim[30], Yongfen Zhu[31], Geraldine Ooi[32], Junping Shi[33], Masato Yoneda[34], Atsushi Nakajima[34], Jing Zhang[35], Monica Lupsor-Platon[36], Bihui Zhong[37], Jeremy F. L. Cobbold[38], Chun-Yan Ye[39], Peter J Eddowes[40], Philip Newsome[41,42,43], Jie Li[44], Jacob George[45], Fangping He[46], Myeong Jun Song[47], Hong Tang[48], Yuchen Fan[49], Jidong Jia[50], Liang Xu[51], Su Lin[52], Yiling Li[53], Zhonghua Lu[54], Yuemin Nan[55], Junqi Niu[56], Xuebing Yan[57], Yongjian Zhou[58], Chenghai Liu[59], Hong Deng[60], Qing Ye[61], Qing-Lei Zeng[62], Lei Li[63], Jing Wang[64], Song Yang[65], Huapeng Lin[66,67], Hye Won Lee[30], Terry Cheuk-Fung Yip[66,67], Céline Fournier-Poizat[68], Grace Lai-Hung Wong[66,67], Grazia Pennisi[15], Angelo Armandi[16], Wen-Yue Liu[69], Ying Shang[17], Marc de Saint-Loup[19], Elba Llop[21], Kevin Kim Jun Teh[22], Carmen Lara-

Romero[23], Amon Asgharpour[24], Sara Mahgoub[70], Mandy Sau-Wai Chan[68], Clemence M. Canivet[19,20], Fanpu Ji[71], Yongning Xin[72], Jin Chai[73], Zhiyong Dong[74], Giovanni Targher[75,76] Christopher D. Byrne[77], Na He[78], Man Mi[1], Feng Ye[2], Vincent Wai-Sun Wong[66,67*], Michael Pavlides[79*], Ming-Hua Zheng[80,81*]

Affiliations:

1. Xi'an Medical University, Xi'an, China;
2. Department of Infectious Diseases, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China;
3. Oxford Centre for Clinical Magnetic Resonance Research, Division of Cardiovascular Medicine, Radcliffe Department of Medicine, University of Oxford, Oxford, UK;
4. Senior Department of Hepatology, The Fifth Medical Center of Chinese PLA General Hospital, Beijing, China;
5. Division of Gastroenterology, Department of Medicine, Faculty of Medicine, Chulalongkorn University and Thai Red Cross, Bangkok, Thailand;
6. Department of Gastroenterology and Hepatology, Saiseikai Suita Hospital, Suita, Japan;
7. Hepatology Unit, Xiamen Hospital of Traditional Chinese Medicine, Xiamen, Fujian, China;
8. UCL Institute for Liver and Digestive Health, Royal Free Hospital, London, UK;

9. Hepatology Unit, Department of Infectious Diseases, Nanfang Hospital, Southern Medical University, Guangzhou, China;

10. Department of Internal Medicine I, University Medical Centre of the Johannes Gutenberg-University Mainz, Mainz, Rhineland-Palatinate, Germany;

11. Department of Medicine II, University Medical Center Homburg, Homburg and University of the Saarland, Saarbrücken, Germany;

12. Gastroenterology and Hepatology Unit, Department of Medicine, Faculty of Medicine, University of Malaya, Malaysia;

13. Department of Hepatology, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangdong Provincial Hospital of Chinese Medicine, Guangzhou, China;

14. Centre d'Investigation de la Fibrose Hépatique, Hôpital Haut-Lévêque, Bordeaux University Hospital, Pessac, and INSERM U1312, Bordeaux University, Bordeaux, France;

15. Sezione di Gastroenterologia, Di.Bi.M.I.S., University of Palermo, Italy；

16. Department of Medical Sciences, Division of Gastroenterology and Hepatology, A.O. Città della Salute e della Scienza di Torino, University of Turin, Turin, Italy；

17. Department of Medicine, Huddinge, Karolinska Institutet, Sweden；

18. Division of Hepatology, Department of Upper GI Diseases, Karolinska University Hospital, Huddinge, Stockholm, Sweden；

19. Hepato-Gastroenterology and Digestive Oncology Department, Angers University

Hospital, Angers, France；

20. HIFIH Laboratory, SFR ICAT 4208, Angers University, Angers, France；

21. Department of Gastroenterology and Hepatology, Hospital Universitario Puerta de Hierro Majadahonda, Madrid, Spain；

22. Department of Gastroenterology and Hepatology, Singapore General Hospital, Singapore；

23. Digestive Diseases Unit and CIBERehd, Virgen Del Rocío University Hospital, Seville, Spain；

24. Stravitz-Sanyal Institute of Liver Disease and Metabolic Health, Department of Internal Medicine, VCU School of Medicine, Richmond, VA, USA；

25. Department of Gastroenterology, Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine, Shanghai Key Lab of Pediatric Gastroenterology and Nutrition, Shanghai, China；

26. Université Paris Cité, UMR1149 (CRI), INSERM, Paris, France; Service d'Hépatologie, Hôpital Beaujon, Assistance Publique-Hôpitaux de Paris (AP-HP), Clichy, France；

27. Division of Gastroenterology & Hepatology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts；

28. Radcliffe Department of Medicine, University of Oxford, Oxford, United Kingdom；

29. Pinnacle Clinical Research, San Antonio, Texas, USA；

30. Department of Internal Medicine, Institute of Gastroenterology, Yonsei University College of Medicine, Seoul, Republic of Korea；

31. Department of Hepatology and Infection, Sir Run Run Shaw Hospital, Affiliated with School of Medicine, Zhejiang University, Hangzhou, China;

32. Centre for Obesity Research and Education, Department of Surgery, Monash University, Melbourne, Australia;

33. Department of Hepatology, The Affiliated Hospital of Hangzhou Normal University, Hangzhou, China;

34. Department of Gastroenterology and Hepatology, Yokohama City University School of Medicine, Yokohama, Japan;

35. The Third Unit, Department of Hepatology, Beijing Youan Hospital, Capital Medical University, Beijing, China;

36. Department of Medical Imaging, Iuliu Hatieganu, University of Medicine and Pharmacy, Regional Institute of Gastroenterology and Hepatology "Prof. Dr. Octavian Fodor", Cluj-Napoca, Romania;

37. Department of Gastroenterology, the First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China;

38. Translational Gastroenterology Unit, University of Oxford, Oxford, UK；

39. Institute for the Study of Liver Diseases, The Third People's Hospital of Changzhou, Changzhou, Jiangsu Province, China;

40. National Institute for Health Research Nottingham Biomedical Research Centre,

Nottingham University Hospitals NHS Trust and University of Nottingham, Nottingham, UK;

41. National Institute for Health Research Biomedical Research Centre at University Hospitals Birmingham NHS Foundation Trust and the University of Birmingham, Birmingham, UK;
42. Centre for Liver and Gastrointestinal Research, Institute of Immunology and Immunotherapy, University of Birmingham, Birmingham, UK;
43. Liver Unit, University Hospitals Birmingham NHS Foundation Trust, Birmingham, UK;
44. Department of Infectious Diseases, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, Nanjing, Jiangsu, China;
45. Storr Liver Centre, Westmead Institute for Medical Research, Westmead Hospital and University of Sydney, NSW, Australia;
46. Department of Hepatobiliary Pancreatic Surgery, Eighth Hospital Affiliated to SunYat-sen University, Futian, Guangdong Province, China;
47. Division of Hepatology, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, Seoul, Korea;
48. Center of Infectious Diseases, West China Hospital, Sichuan University, Chengdu, China;
49. Department of Hepatology, Qilu Hospital of Shandong University, Jinan, China;
50. Liver Research Center, Beijing Friendship Hospital, Capital Medical University,

Beijing, China;

51. Department of Hepatology, Tianjin Second People's Hospital, Tianjin, China;

52. Department of Hepatology, Hepatology Research Institute, The First Affiliated Hospital of Fujian Medical University, Fuzhou, China;

53. Department of Gastroenterology, First Affiliated Hospital of China Medical University, Shenyang, China;

54. Clinical Laboratory Center, The Fifth People's Hospital of Wuxi, Wuxi, Jiangsu, China;

55. Department of Traditional and Western Medical Hepatology, Hebei Medical University Third Hospital, Shijiazhuang, China;

56. Department of Hepatology, Center of Infectious Diseases and Pathogen Biology, The First Hospital of Jilin University, Changchun, China;

57. Department of Infectious Diseases, The Affiliated Hospital of Xuzhou Medical University, Xuzhou, Jiangsu, China;

58. Department of Gastroenterology and Hepatology, Guangzhou First People's Hospital, School of Medicine, South China University of Technology, Guangzhou, China;

59. Institute of Liver Diseases, Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai, China;

60. Department of lnfectious Diseases, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China；

61. Department of Hepatology of The Third Central Hospital of Tianjin, Tianjin, China;

62. Department of Infectious Diseases, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China;

63. Department of lnfectious Diseases, The First Affiliated Hospital of Anhui Medical University, Hefei, China;

64. Department of Hepatobiliary Diseases, Affiliated Traditional Chinese Medicine Hospital of Southwest Medical University, Luzhou, Sichuan Province, China;

65. Center of Hepatology, Beijing Ditan Hospital, Capital Medical University, Beijing, China;

66. Medical Data Analytics Centre, Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Hong Kong, China；

67. State Key Laboratory of Digestive Disease, Institute of Digestive Disease, The Chinese University of Hong Kong, Hong Kong, China；

68. Echosens, Paris, France；

69. Department of Endocrinology, First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China；

70. National Institute for Health Research, Biomedical Research Centre at University Hospitals Birmingham NHS Foundation Trust and the University of Birmingham, Birmingham, United Kingdom；

71. Department of Infectious Diseases, The Second Affiliated Hospital of Xi'an

Jiaotong University, Xi'an, China;

72. Department of Infectious Diseases, Qingdao Municipal Hospital Affiliated to Qingdao University, Qingdao, China;

73. Department of Gastroenterology, Southwest Hospital, Army Medical University, Chongqing, China;

74. Department of Metabolic and Bariatric Surgery, The First Affiliated Hospital of Jinan University, Guangzhou, China;

75. Department of Medicine, University of Verona, Verona, Italy;

76. Metabolic Diseases Research Unit, IRCCS Sacro Cuore - Don Calabria Hospital, Negrar di Valpolicella, Italy;

77. Southampton National Institute for Health and Care Research Biomedical Research Centre, University Hospital Southampton and University of Southampton, Southampton General Hospital, Southampton, UK;

78. Department of Gastroenterology, The First Affiliated Hospital of Xi'an Medical University, Xi'an, China;

79. Oxford Centre for Clinical Magnetic Resonance Research, University of Oxford, Oxford, UK;

80. MAFLD Research Center, Department of Hepatology, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China;

81. Key Laboratory of Diagnosis and Treatment for The Development of Chronic Liver Disease in Zhejiang Province, Wenzhou, Zhejiang, China.

[#]These authors contributed equally to the study.

***Corresponding authors:**

Ming-Hua Zheng, M.D., Ph.D., MAFLD Research Center, Department of Hepatology, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China. Tel: 86-577-55579611; Fax: 86-577-55578522; E-mail: zhengmh@wmu.edu.cn

Dr. Michael Pavlides, Oxford Centre for Clinical Magnetic Resonance Research (OCMR), University of Oxford, Oxford, UK; E-mail: michael.pavlides@cardiov.ox.ac.uk

Prof Vincent Wong, Department of Medicine and Therapeutics, Prince of Wales Hospital, Shatin, Hong Kong, China. Tel: +852 35054205; Email: wongv@cuhk.edu.hk

Total word count: 3855 words

Number of figures/supplementary figures: 3/6

Number of tables/supplementary tables: 1/12

Author contributions to this manuscript:

MHZ, and GF were involved in study design, data interpretation, and verification. GF performed data analysis. GF wrote the manuscript. Data collection was done by FEM, DJ, ST, TO, TS, HL, ET, JJC, JMS, CL, SM, WKC, XC, Ad, VdL, SP, EB, HH, JB, JLC, GBG, RGD, AJS, JGF, LC, ML, SAH, MRG, SUK, YFZ, GO, JPS, MY, AN, JZ, MLP, BZ, JFLC, CYY, PJE, PN, JL, JG, FPH, MJS, HT, YF, JDJ, LX, SL, YLL, ZHL, YMN, JQN, XBY, YJZ, CHL, HD, QY, QLZ, LL, JW, SY, HL, HWL, TCFY, CFP, GLHW, GP, AA, WYL, YS, MDSL, EL, KKJT, CLR, AA, SM, MSWC, CMC, FPJ, YNX, JC, ZYD, GT, CDB, NH, FY, MP, MHZ. GT, CDB, VWSW, and MP conducted critical revision and writing of the manuscript. All authors reviewed and commented on the manuscript and approved the final version.

Grants Support:

This work was supported by grants from the National Natural Science Foundation of China (82070588), National Key R&D Program of China (2023YFA1800801), High Level Creative Talents from Department of Public Health in Zhejiang Province (S2032102600032), Science and Technology project of Shaanxi Province (2023-YBSF-385), Natural Science Basic Research Program of Shaanxi province(2020JM-399 and 2023-JC-YB-699), Scientific Research Plan Project of Shaanxi Provincial Department of Education (23JK0646) and Project of New Century 551 Talent Nurturing in Wenzhou. CDB is supported in part by the Southampton National Institute for Health Research Biomedical Research Centre, UK (NIHR203319).

Conflicts of Interest:

JMS serves as a consultant for Akero, Alentis Therapeutics, Astra Zeneca, Apollo Endosurgery, Boehringer Ingelheim, GSK, Ipsen, Inventiva Pharma, Madrigal, MSD, Northsea Therapeutics, Novartis, Novo Nordisk, Pfizer, Roche, Sanofi, and Siemens Healthineers. He has received research funding from Gilead Sciences, Boehringer Ingelheim and Siemens Healthcare GmbH. He holds stock options in AGED diagnostics and Hepta Bio. JMS has also received speaker honorarium from Gilead Sciences, Advanz, Echosens, and MedPublico GmbH. WKC is a consultant or advisory board member for Roche, Abbvie, Boehringer Ingelheim and Novo Nordisk and a speaker for Novo Nordisk, Abbott, Echosens, Viatris and Hisky Medical. VDL has served as a consultant or advisory committee member for Gilead Sciences, Intercept, Alfasigma, Orphalan and Mindray and a speaker for AbbVie, Echosens, Gilead Sciences, Hologic, Tillotts, Orphalan and Janssen. FPJ reports lecture fees from Gilead Sciences, MSD and Ascletis and is a consultant for Gilead and MSD. CDB has received grant support from Echosens. SP served as a speaker or advisor for AbbVie, Echosens, MSD, Novo Nordisk, Pfizer, and Resalis. EB served as a consultant for Boehringer, MSD, Novo Nordisk, and Pfizer; and a speaker for MSD, Novo Nordisk, and Madrigal. She received research grants from Gilead Sciences. HH served as a consultant for AstraZeneca; and a hepatic events adjudication committee member for KOWA and GW Pharama. His institution has received research funding

from AstraZeneca, Echosens, Gilead Sciences, Intercept, MSD, and Pfizer. JB served as a consultant for AstraZeneca, Echosens, Intercept, and Siemens; a speaker for AbbVie, Gilead Sciences, Intercept, and Siemens; and an advisory board member for Bristol-Myers-Squibb, Intercept, Pfizer, MSD, and Novo Nordisk. His institution has received research funding from Diafir, Echosens, Intercept, Inventiva, and Siemens. JLC served as a consultant and speaker for Echosens, Gilead Sciences, and AbbVie. GB-BG served as a consultant for Roche and Ionis Pharmaceuticals; and a speaker for Echosens, Viatris, Abbott and Novo Nordisk. AJS servedb as a consultant for Akero, Allergan, Alnylam Pharmaceuticals, Amgen Inc, AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, Genentech, Gilead Sciences, Histoindex, Intercept Pharmaceuticals, Inventiva, Madrigal, Merck, Novartis, Novo Nordisk, Pfizer, Poxel, Salix Pharmaceuticals, Siemens, Sun Pharmaceutical Industries Inc, Terns, and Valeant Pharmaceuticals; and a data safety monitoring board or advisory board member for Bard Peripheral Vascular Inc, NGM Biopharmaceuticals, and Sequana. He has received research funding from Albireo, Allergan, Echosens, Eli Lilly, Gilead Sciences, Intercept Pharmaceuticals, Mallinckrodt LLC, Merck, Novo Nordisk, Perspectum, Pfizer, Salix Pharmaceuticals, and Zydus; and holds the stocks of Durect, Exhalenz, Gen t, and Tiziana. MR-G served as a consultant for Siemens; and a speaker for Siemens and Echosens. He has received research funding from Siemens, Echosens, and Novo Nordisk. PN served as a consultant for Novo Nordisk, Boehringer Ingelheim, Gilead Sciences, Intercept, Poxel Pharmaceuticals, Pfizer,

BMS, Eli Lilly, Madrigal, and GSK; and a speaker for Novo Nordisk and AiCME. He has received research funding from Novo Nordisk. LC served as a consultant for Echosens, Novo Nordisk, Madrigal, MSD, Pfizer, Sagimet, and Siemens Healthlineers; and a speaker for Echosens, Gilead, Inventiva, and Novo Nordisk. SH served as a consultant for Akero Therapeutics, Aligos, Altimmune, Arrowhead, Bluejay Therapeutics, Boxer Capital, Chronwell, Echosens, Enyo, Foresite Labs, Galectin, Galecto, Gilead, GSK, Hepagene, Hepion, Hepta Bio, HistoIndex, Humana, Intercept, Ionis, Madrigal, Medpace, NeuroBo Pharmaceuticals, Northsea, Novo Nordisk, Perspectum, Pfizer, Sonic Incytes, Sagimet, Terns, and Viking; an advisory board member for Akero, Altimmune, Arrowhead,BVF Partners, Chronwell, Echosens, Foresite Labs, Galectin, Genfit, Gilead, Hepion, Hightide, HistoIndex, Humana, Intercept, Madrigal, Medpace, Metacrine, NGM Bio, Northsea, Novo Nordisk, PathAI, Poxel, Sagimet Biosciences, Sonic Incytes Medical Corp, and Terns. He holds the stocks of Akero Therapeutics, Chronwell, Cirius, Galectin, Genfit, Hepion, Hepta Bio, HistoIndex, Metacrine, NGM Bio, Northsea, and Sonic Incytes; and grants and contracts from Akero Therapeutics, Altimmune, Axcella, BMS, Corcept, Cymabay, Enyo, Galectin, Genentech, Genfit, Gilead, GSK, Hepion, Hightide, Immuron, Intercept, Inventiva, Ionis, Madrigal, NGM Bio, Novartis, Novo Nordisk, Northsea, Pfizer, Poxel, Sagimet, Terns, and Viking. CF-P is an employee of Echosens. GL-HW served as a consultant for AstraZeneca, Gilead Sciences, and Janssen; and a speaker for Abbott, Abbvie, Bristol-Myers Squibb, Echosens, Furui,

Gilead Sciences, and Roche. She has received research funding from Gilead Sciences. MS-WC is an employee of Echosens. VW-SW served as a consultant or advisory board member for AbbVie, Boehringer Ingelheim, Echosens, Gilead Sciences, Intercept, Inventiva, Novo Nordisk, Pfizer, Sagimet Biosciences, TARGET PharmaSolutions, and Visirna; and a speaker for Abbott, AbbVie, Gilead Sciences, Novo Nordisk, and Unilab. He has received a research grant from Gilead Sciences, and is a co-founder of Illuminatio Medical Technology. The other authors have no conflicts of interest to declare.

Acknowledgments

The authors thank the members of the CHESS-MAFLD consortium and the LITMUS investigators for their coordination.

Abbreviation list: AUC, area under the curve; AST, aspartate aminotransferase; CAP, controlled attenuation parameter; FAST, FibroScan-AST score; LSM, liver stiffness measurements; MAFLD, metabolic dysfunction-associated fatty liver disease; MAS, MAFLD activity score; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease; NHANES, Nutrition Examination Survey; NPV, negative predictive value; PPV, positive predictive value; ROC, receiver operator characteristic; SCr, serum creatinine, T2DM, type 2 diabetes mellitus.

Abstract

Background & Aims: Metabolic dysfunction-associated steatohepatitis (MASH) and fibrotic MASH are significant health challenges. This multi-national study aimed to validate the acMASH index (including serum creatinine and aspartate aminotransferase concentrations) for MASH diagnosis and develop a new index (acFibroMASH) for non-invasively identifying fibrotic MASH and exploring its predictive value for liver-related events (LREs).

Methods: We analyzed data from 3,004 individuals with biopsy-proven metabolic dysfunction-associated fatty liver disease (MAFLD) across 29 Chinese and nine international cohorts to validate the acMASH index and develop the acFibroMASH index. Additionally, we utilized the independent external data from a multi-national cohort of 9,034 patients with MAFLD to examine associations between the acFibroMASH index and the risk of LREs.

Results: In the pooled global cohort, the acMASH index identified MASH with an AUROC of 0.802 (95%CI 0.786-0.818). The acFibroMASH index (including the acMASH index *plus* liver stiffness measurement) accurately identified fibrotic MASH with an AUROC of 0.808 in the derivation cohort and 0.800 in the validation cohort. Notably, the AUROC for the acFibroMASH index was 0.835 (95% CI 0.786-0.882), superior to that of the FAST score at 0.750 (95% CI 0.693-0.800, P<0.01) in predicting the 5-year risk of LREs. Patients with acFibroMASH >0.39 had a higher

risk of LREs than those with acFibroMASH <0.15 (adjusted-hazard ratio: 11.23 95%CI 3.98-31.66).

Conclusions: This multi-ethnic study validates the acMASH index as a reliable, non-invasive test for identifying MASH. The newly proposed acFibroMASH index is a reliable test for identifying fibrotic MASH and predicting the risk of LREs.

Keywords: Fibrotic metabolic-associated steatohepatitis, Diagnosis, Liver-related events

Introduction

Metabolic dysfunction-associated fatty liver disease (MAFLD) is the leading cause of chronic liver diseases worldwide, affecting up to ~30% of the global adult population.[1] As part of the disease continuum in MAFLD, metabolic dysfunction-associated steatohepatitis (MASH) is associated with faster progression to advanced fibrosis and increased all-cause and liver-related mortality. MASH and its progressive form, fibrotic MASH, are significant global health issues, posing a substantial burden on the global healthcare systems.[2]

Fibrotic MASH (also known as at-risk MASH) is characterized by an elevated histological activity score and fibrosis stage F ≥2 and strongly predicts future liver-related complications.[3] Existing non-invasive tests are primarily designed for identifying advanced liver fibrosis (stage F3 or cirrhosis) and show limited accuracy for earlier stages of the disease, such as fibrotic MASH.[3, 4] Hence, we hypothesize that constructing the fibrotic MASH index should encompass MASH status and fibrosis parameters. Our Team has recently developed a test named the acMASH index using serum creatinine (SCr) and aspartate aminotransferase (AST) concentrations for the non-invasive identification of MASH.[5] The acMASH index is a convenient diagnostic tool for MASH validated across multiple cohorts. Liver stiffness measurement (LSM), obtained by vibration-controlled transient elastography techniques, can quantify the

severity of liver fibrosis and has been used as an essential foundational parameter in various fibrotic MASH scores. Therefore, we speculate that a new non-invasive diagnostic model for fibroMASH could be constructed by combining the acMASH index (reflecting the MASH status) with LSM values (reflecting the fibrosis stage). Moreover, in patients with MAFLD, there is a close relationship between the severity of liver fibrosis and the risk of developing liver-related events (LREs).

Thus, this multi-national and multi-ethnic study of patients with MAFLD aimed to develop and validate a simple and reliable test termed the acFibroMASH index for the non-invasive identification of fibrotic MASH and to explore its prognostic role for predicting future LREs. As a secondary objective, we aimed to validate the acMASH index for the non-invasive identification of MASH.

Methods

Data Source and Patients

We performed a multidimensional study including three international multicenter cohort datasets. All patients' data were anonymized to protect patient privacy.

The first database was used to validate the diagnostic performance of acMASH. Consecutive participants in the first cohort dataset were recruited from 29 medical centers across China (from December 2012 to January 2023) and nine international cohorts (from January 2006 to January 2017, **eMethod 1**) with biopsy-proven fatty liver disease diagnosed according to the international consensus recommendations published and endorsed by the Asian Pacific Association for the Study of Liver Disease (APASL), the Middle East and North African consensus, and the Chinese Society of Hepatology.[6-10] The sample size of each cohort in the first dataset is shown in **Supplementary Table 1**. Three pan-national liver associations recently suggested a new terminology and definition: metabolic dysfunction-associated steatotic liver disease (MASLD).[11] The MAFLD patient population in the present study met the diagnostic criteria for MASLD. In this study, the diagnostic criteria of MAFLD are based on evidence of hepatic steatosis on liver histology, combined with evidence of metabolic dysfunction.[7] We also used the National Health and Nutrition Examination Survey (NHANES) study to explore the associations between acMASH and the risk of all-cause and cause-specific mortality (**eMethod 2**).

The second dataset was derived from the first dataset after excluding subjects who did not have vibration-controlled transient elastography (VCTE) measures. This dataset comprised a derivation cohort of Chinese patients with biopsy-proven MAFLD from

Wenzhou (n=218) and an external validation international cohort of patients with MAFLD (n=473). This dataset was used to develop and validate the acFibroMASH index.

The third dataset was used to explore the predictive value of the newly proposed acFibroMASH index for the future risk of LREs and derived from a cohort of individuals diagnosed with MAFLD from February 2004 to January 2023, who underwent VCTE assessments across 16 sites in the United States, Europe, and Asia. The dataset included adult patients (18 years or older) with hepatic steatosis, confirmed through either liver biopsy, imaging modalities (including ultrasound, CT, or MRI), or a controlled attenuation parameter (CAP) reading of 248 dB/m or more on VCTE. [12]

Clinical Assessment and Data Collection

We collected the clinical, biochemical, and liver histological characteristics of the global cohort. The acMASH index was calculated for each participant and was derived using the following formula: acMASH = AST (U/L)/SCr (μmol/L)*10.[5] An acMASH index below 4.15 was used to rule out MASH, and an acMASH index above 7.73 was used to rule in MASH.[5] For the development of the acFibroMASH index for identifying fibrotic MASH, we considered that the parameters included in the

FibroScan-AST score (FAST) score, i.e., a VCTE-based score developed for the non-invasive diagnosis of fibrotic MASH, including LSM, CAP and serum aminotransferase levels, are essential parameters for predicting fibrotic MASH.[13, 14] The FAST score uses the following formula: FAST = $\frac{e^{-1.65+1.07\times\ln(LSM)+2.66\times10^{-8}\times CAP^3-63.3\times AST^{-1}}}{1+e^{-1.65+1.07\times\ln(LSM)+2.66\times10^{-8}\times CAP^3-63.3\times AST^{-1}}}$. The FAST score's parameters are combined individually with the acMASH index to find the optimal combination method to construct the acFibroMASH index. In the Results section, we reported the specific formula of the acFibroMASH index.

Definition of liver-related events

LREs were defined as a composite endpoint, including the occurrence of hepatocellular carcinoma, liver decompensation (ascites, variceal bleeding, hepatic encephalopathy, or hepatorenal syndrome), liver transplantation, or liver-related death. We have compared the acFibroMASH index to the FAST score in diagnosing fibrotic MASH and predicting the future risk of LREs.

Liver histology

Experienced liver pathologists assessed liver biopsies at the centers where the studies were conducted. All liver biopsy specimens were scored according to the MAFLD Activity Score (MAS) staging and grading systems. Fibrosis stage was defined as F0

(absence of fibrosis), F1 (perisinusoidal or periportal fibrosis), F2 (perisinusoidal and portal/periportal fibrosis), F3 (bridging fibrosis), and F4 (cirrhosis). MAFLD was diagnosed by identifying >5% steatosis on liver biopsy combined with at least one of the following three metabolic abnormalities: overweight/obesity, presence of type 2 diabetes, or evidence of metabolic dysregulation.[7] Definitive MASH was defined as a score equal to or greater than 5, with a minimum score of 1 for each of the following categories: steatosis, lobular inflammation, and ballooning.[15] Significant fibrosis was defined as a fibrosis stage ≥F2. Subjects with elevated MAS (MAS ≥4), a fibrosis stage ≥F2, and at least 1 point in each of the components of the MAFLD activity score were classified as having fibrotic MASH.[16]

Data Analysis

Continuous variables were expressed as means ± standard deviation (SD) or medians and interquartile range (IQR) as appropriate. Categorical variables were presented as proportions. The diagnostic performance of the acMASH index in identifying MASH was evaluated using the area under the receiver operating characteristic (AUROC) curve, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). We used various methods to validate additional factors (besides acMASH) associated with fibrotic MASH, including multivariable regression analysis, AUROC and Spearman's correlation analyses. Specifically, we used the

logistic regression analysis to combine the acMASH index with each parameter of the FAST score and variables screened by multivariable regression analysis individually, with the Delong test to compare the AUROC of each regression model. The model with the highest AUROC was considered the optimal combination method for the acFibroMASH index. Additionally, Spearman's correlation analysis was utilized to determine which parameters derived from the FAST score and multivariable regression analysis were most strongly correlated to the stage of fibrosis. Fibrosis stage is a crucial factor in assessing fibrotic MASH, aside from the MASH status. The diagnostic performance of the acFibroMASH index for predicting fibrotic MASH was assessed using the AUROC curve, sensitivity, specificity, PPV, and NPV. Cut-offs of the acFibroMASH index for sensitivity (≥0.90) and specificity (≥0.90) were obtained in the derivation cohort. These cut-off values were subsequently applied to the external cohorts. The association between the acMASH index and the future risk of LREs was assessed using hazard ratios (HR) and 95% confidence intervals (CI) derived from Cox proportional hazards regression models. Survival analyses were performed using the Kaplan-Meier survival method. Moreover, to ensure the stability of the results, we also employed a competing risk regression model. For the primary outcome, non-liver-related death was treated as a competing event. The method for time-dependent receiver operating characteristic (ROC) analysis we took was based on a cumulative/dynamic approach.[17] Statistical analyses were performed using

STATA version 17.0 and R software version 4.0.0. All statistical tests were two-sided, and a P-value of less than 0.05 was considered significant.

Results

Development and validation of the diagnostic performance of the acFibroMASH index for fibrotic MASH

Before establishing the acFibroMASH index, we validated the diagnostic accuracy of the acMASH index in the first database. The detailed findings can be found in **eResult 1** (**Supplementary Tables 1-6**, **Supplementary Figure 1A, Supplementary Figure 2, and Supplementary Figure 3**). Subsequently, we used the second dataset, which included a derivation cohort of 218 Chinese MAFLD patients from Wenzhou and an external validation cohort of 473 international MAFLD patients with available LSM data (**Supplementary Figure 1B**). The clinical, biochemical, and liver histology characteristics of patients in the second dataset are summarized in **Supplementary Table 7.** The results of the multivariable logistic regression showed that only LSM (OR 1.372 [95% CI 1.167-1.613]) was statistically correlated with fibrotic MASH among the potential parameters (**Supplementary Table 8**). We considered the combination of the acMASH index with other indicators, referring to parameters used in the FAST score modeling process, including VCT-measured LSM, CAP, serum AST, ALT, and AST/ALT ratio as well as the index (LSM) derived from the

multivariable logistic regression analysis. Further combining the acMASH index with the indicators mentioned above, we discovered that the model, constructed by combining the acMASH index with VCTE-measured LSM (acFibroMASH model), had the highest AUROC (0.808 [95%CI 0.748-0.869]), outperforming all other combination forms (P<0.05, **Figure 1A**), with the decision curve also surpassing all other combinations (**Figure 1B**), thus indicating the best positive net benefit of combining the acMASH index with VCTE-measured LSM. The calibration curve of acFibroMASH, by combining the acMASH index with VCTE-measured LSM, is shown in **Figure 1C,** and the curve shows a good agreement. We also found that the indicators most closely related to fibrosis stage, in order of relevance, were LSM (r=0.483, P <0.01), acMASH (r = 0.397, P <0.01), AST (r=0.371, P<0.01), ALT (r=0.285, P <0.01), and CAP (r=0.142, P=0.037), with the AST/ALT ratio not showing a correlation with the stage of fibrosis (P=0.564) (**Figure 1D**).

The specific formula was as follows: acFibroMASH = $e^{-3.956 +0.305*LSM +0.065*acMASH}$/ $(1+e^{-3.956 +0.305*LSM +0.065*acMASH})$. A cut-off for the index <0.15 gave a sensitivity of 90% and an NPV of 93% for ruling out fibrotic MASH. Conversely, a cut-off of the acFibroMASH index >0.39 gave a specificity of 90% and a PPV of 60% for ruling-in fibrotic MASH (**Table 1**). In the derivation cohort, we found that the AUROC of the acFibroMASH index (0.808 [95%CI 0.748-0.869]) was significantly better (*P*=0.040)

than that of the FAST score (0.764 [95%CI 0.694-0.834]), as reported in **Figure 2A.** The plots of DCA for the acFibroMASH and FAST scores are shown in **Figure 2B**, indicating the positive net benefit of the established models. A cut-off of the FAST score ≤0.35 gave a sensitivity of 83% and an NPV of 89% for ruling out fibrotic MASH. Conversely, a cut-off of the FAST score ≥0.67 gave a specificity of 91% and a PPV of 62% for ruling-in fibrotic MASH. In the context of the "indeterminate gray zone", the acFibroMASH score was substantially similar to the FAST score (41% vs. 37%, p > 0.05, **Supplementary Figure 4**).

In the external validation cohort (n=473), the diagnostic performance of the acFibroMASH index for identifying fibrotic MASH was good, with an AUROC of 0.800 (95%CI 0.758-0.839, **Figure 2C**). A cut-off of the acFibroMASH index <0.15 gave a sensitivity of 92% and an NPV of 90% for ruling out fibrotic MASH. A cut-off of the acFibroMASH index >0.39 gave a specificity of 83% and a PPV of 70% for ruling-in fibrotic MASH **(Table 1).** In the global cohort (n=691), the AUROC of acFibroMASH was 0.806 (95%CI 0.773-0.840, **Figure 2C**). The AUROCs of acFibroMASH in different patient subgroups are shown in **Supplementary Table 9.** A cut-off of the acFibroMASH index <0.15 gave a sensitivity of 92% and an NPV of 92% for ruling out fibrotic MASH. A cut-off of the acFibroMASH index >0.39 gave a specificity of 86% and a PPV of 68% for ruling in fibrotic MASH (**Figure 2D**).

Predictive value of the acFibroMASH index for future LREs

After excluding 8,915 patients according to the exclusion criteria, a total of 9,034 patients with MAFLD were included in the analysis to explore the value of the acFibroMASH index for predicting future LREs (**Supplementary Figure 5**). Throughout the entire follow-up period, 136 patients (1.5%) experienced LREs. For predicting the risk of LREs over three years of follow-up, the AUROC for acFibroMASH was 0.855 (95% CI 0.790-0.913), superior to that of the FAST score at 0.762 (95% CI 0.692-0.827, P<0.001 for comparison, **Figure 3A**). For predicting the LREs over five years of follow-up, the AUROC for acFibroMASH was 0.835 (95% CI 0.786-0.882), superior to that of FAST at 0.750 (95% CI 0.693-0.800, P<0.001 for comparison, **Figure 3B**). Cumulative incidence rates of LREs stratified by acFibroMASH are shown in **Figure 3C.** Patients with acFibroMASH > 0.39 had a markedly higher risk of LREs than those with acFibroMASH <0.15 [HR of 21.092 (95% CI 12.882-34.553) in the age- and sex-adjusted model, HR of 20.687 (12.502-34.235) in the model 1 adjusted for age, sex, hypertension, diabetes, and BMI, and HR of 11.231 (3.984-31.657) in model 2 additionally adjusted for serum GGT, ALT, total cholesterol, triglycerides, HbA1c, glucose, and platelet count] (**Supplementary Table 10**). Similarly, the competing risk regression model showed that patients with acFibroMASH > 0.39 had an increased risk of developing LREs with an HR of 19.82

(95% CI 11.92-32.98), HR of 19.45 (95% CI 11.57-32.69), and HR of 10.88 (95% CI 3.82-31.03) across the three adjusted regression models mentioned above (**Supplementary Table 11**). In addition, the relationship between the acMASH index and LREs is shown in **eResult 2** (**Supplementary Table 12, Supplementary Figure 6**).

Discussion

The main and novel findings of this multi-national, multi-ethnic study show that the acMASH and acFibroMASH indices provide an efficient way to non-invasively identify patients with MASH and fibrotic MASH, reducing the need for unnecessary liver biopsies in patients not likely to have significant liver disease. The acMASH index had good diagnostic performance for identifying MASH with an AUROC value above 0.80 in the pooled China, international and global cohorts. The acFibroMASH index also showed good diagnostic performance for the non-invasive identification of fibrotic MASH. Finally, the acFibroMASH index had prognostic value in predicting the future risk of LREs in adults with at-risk MASH (especially those with an index >0.39).

Despite the escalating global burden of MAFLD, optimizing healthcare strategies for efficient screening, referral, assessment, and management remains to be fully

established.[4] Non-invasive tests that utilize standard laboratory parameters have a significant advantage as a screening method in primary healthcare and non-hepatology settings. Here, we demonstrate the utility of the acMASH index as a reliable diagnostic tool for non-invasively identifying MASH across diverse racial and geographic populations. Since the acMASH index is simple to calculate and can be incorporated into practice software, it provides a convenient bedside test for diagnosing MASH in primary care.

The newly developed acFibroMASH index is noteworthy because it showed high accuracy for non-invasively identifying fibrotic MASH with AUROCs around 0.80 in both the derivation and validation cohorts. The index's accuracy appears better than the FAST score, thus promoting its potential as a superior non-invasive diagnostic tool. Serum creatinine concentration was included in the acFibroMASH index due to its independent association with MASH, its association with the histological NAS scores, and the NAFLD's impact on the urea cycle and remethylation, affecting creatine synthesis.[5] In our study, the AST parameter was not again incorporated into the acFibroMASH index. This decision was based on the results of the multi-factor and ROC curve analyses, as well as the fact that the acFibroticMASH index already includes the AST parameter, thus reducing the risk of collinearity by excluding AST as a further additional factor in the regression model.

Notably, the results of our study also affirm the predictive value of the acFibroMASH index for adverse clinical outcomes. Specifically, patients with the acFibroMASH index >0.39 had an increased risk of developing LREs in the long term. This finding has important clinical implications for decision-making in liver disease management and the long-term prognosis of these patients. Adults with an acMASH index >0.39 may already be potential patients with fibrotic MASH, while fibrotic MASH is associated with an increased future risk of LREs.

Despite these promising results, the current study has some important limitations. Firstly, using liver biopsy as a reference standard introduces the potential for sampling errors, given the heterogeneity of MASH and fibrosis within the liver. Secondly, while the acFibroMASH index outperformed the FAST score in the Chinese MAFLD cohort from Wenzhou, we could not validate this result in an external cohort. This limitation arises from a lack of the necessary FibroScan-derived parameters (CAP) to calculate the external cohorts' FAST score. The lack of comparison with other non-invasive tests/biomarkers of fibrotic MASH (e.g., NIS2+, NIS4, MACK-3, MAST, MEFIB) may also be another limitation of our study. Thirdly, serum creatinine concentrations may be influenced by various factors, and thus, the impact of these factors should be considered when using our proposed model for calculating the acFibroMASH.

Furthermore, central liver biopsy reading cannot be implemented due to geographical and resource limitations. Meanwhile, we are planning to achieve a unified pathology center to review liver biopsy samples in future studies, thereby strengthening the validity of our research outcomes. Finally, this study is limited by the loss to follow-up at the endpoint and insufficient follow-up time, which may impact the research results. Lastly, the third dataset, which was used to explore the predictive value of acFibroMASH index for the future risk of LREs, lacks liver biopsy data, especially the pathological information on fibrosis grades. Consequently, it is not possible to accurately describe the proportion of patients with fibrotic MASH in the fibroMASH categories.

In conclusion, the results of this multi-national, multi-ethnic study show that the acMASH and acFibroMASH indices hold promise in the non-invasive identification of MASH and fibrotic MASH. Future studies should seek to validate and/or refine these non-invasive indices, increasing their precision and applicability and exploring their integration into routine clinical practice. Moreover, further prospective studies are warranted to corroborate our findings and establish the prognostic role of the acMASH and acFibroMASH indices in predicting extra-hepatic disease outcomes.

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Table legend

Table 1. Performance of the acFibroMASH index for the non-invasive identification of fibrotic MASH according to liver histology in the derivation cohort and external validation cohort as well as in the pooled global cohort.

Figure legends

Figure 1. The construction process of the acFibroMASH index in the derivation cohort.

(A) ROC curves of the combinations of the acMASH index with different indicators. (B) Decision curves of the combinations of the acMASH index with different indicators. (C) The calibration curve of acFibroMASH index (D) Analysis of the correlation between different parameters and the stage of fibrosis.

Figure 2. Diagnostic performance of the acFibroMASH index for the non-invasive identification of fibrotic MASH (or at-risk MASH).

(A) ROC curves of the acFibroMASH and FAST indices in the derivation cohort. (B) Decision curves of the acFibroMASH and FAST indices in the derivation cohort. (C) ROC curves of the acFibroMASH index in the derivation, validation and global cohorts. (D) Performance of the acFibroMASH index in the global cohorts (N=691) using a dual cut-off approach.

Figure 3. Predictive value of the acFibroMASH index for the risk of developing liver-

related events (LREs)

(A) AUROC for the prediction of a 3-year-risk of LREs by the acFibroMASH and FAST indices. (B) AUROC for the prediction of a 5-year-risk of LREs by the acFibroMASH and FAST indices. (C) Cumulative incidence rates of LREs stratified by the acFibroMASH indeex. Group 0, acFibroMASH <0.15; Group 1, 0.39 ≥ acFibroMASH ≥0.15; Group 3, acFibroMASH >0.39.