remained significant even after further adjustment for the indicators of the metabolic syndrome, including waist circumference, systolic blood pressure, diastolic blood pressure, triglyceride, HDL cholesterol and fasting plasma glucose, the adjusted hazard ratio (95% CI) was 2.21 (1.42–3.44). These results indicated that subclinical hypothyroidism is an independent factor that predicts the development of NAFLD.

A limitation of this study is that ultrasound examination is not sensitive enough to detect mild steatosis. However, ultrasonography is the most commonly used in epidemiological surveys of NAFLD. Ultrasonography has the advantage of being non-invasive, safe, widely available, and sensitivity for detecting hepatic steatosis is acceptable. Another limitation is that the causal relationship between subclinical hypothyroidism and development of NAFLD could not been drawn by this study. Further evidence is needed to clarify this issue.

Taken together, our prospective case-control study provided evidence that subclinical hypothyroidism is a significant factor associated with NAFLD development. This finding may have important clinical implications for disease therapy and prevention.

Conflict of interest

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

Financial support

This study was supported by National Natural Science Foundation of China (Nos. 31070315, 81170378, and 81100278), National Science and Technology Support Plan Project (No. 2012BAI06B04), and National Key Basic Research Development Program (No. 2012CB524905).

References

[1] Chung GE, Kim D, Kim W, Yim JY, Park MJ, Kim YJ, et al. Non-alcoholic fatty liver disease across the spectrum of hypothyroidism. J Hepatol 2012;57:150–156.
[2] Xu C, Yu C, Xu L, Miao M, Li Y. High serum uric acid increases the risk for nonalcoholic Fatty liver disease: a prospective observational study. PLoS One 2010;5:e11578.
[3] Yu C, Xu C, Xu L, Yu J, Miao M, Li Y. Serum proteomic analysis revealed diagnostic value of hemoglobin for nonalcoholicfatty liver disease. J Hepatol 2012;56:241–247.
[4] Surks MI, Ortiz E, Daniels GH, Sawin CT, Col NF, Cobin RH, et al. Subclinical thyroid disease: scientific review and guidelines for diagnosis and management. JAMA 2004;291:228–238.
[5] Rodondi N, Newman AB, Vittinghoff E, de Rekeneire N, Satterfield S, Harris TB, et al. Subclinical hypothyroidism and the risk of heart failure, other cardiovascularevents, and death. Arch Intern Med 2005;165:2460–2466.
[6] Bedogni G, Miglioli L, Masutti F, Tiribelli C, Marchesini G, Bellentani S. Prevalence of and risk factors for nonalcoholic fatty liver disease: the Dionysos nutrition and liver study. Hepatology 2005;42:44–52.
[7] Cerda C, Perez-Ayuso RM, Riquelme A, Soza A, Villaseca P, Sir-Petermann T, et al. Nonalcoholic fatty liver disease in women with polycystic ovary syndrome. J Hepatol 2007;47:412–417.

Lei Xu[†]
Department of Gastroenterology, Ningbo No. 1 Hospital, Ningbo 315010, China
Department of Gastroenterology, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, China

Han Ma[†]
Department of Gastroenterology, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, China

Min Miao
Department of Internal Medicine, Zhenhai Lianhua Hospital, Ningbo 315010, China

Youming Li[*]
Department of Gastroenterology, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, China
[*]Corresponding author. *E-mail address:* xiaofu@zju.edu.cn
[†]*These authors contributed equally to this work.*

Regulatory T cell defects in adult autoimmune hepatitis

To the Editor:

Peiseler *et al.* report that FOXP3^{pos} regulatory T cells (Treg) in adult patients with autoimmune hepatitis (AIH) are "*fully functional and not reduced in frequency*" [1].

We wish to make a few comments.

Quoting previous literature on Tregs in AIH [2,3], the authors state that most information has been obtained in paediatric cases. We have published data on 47 adult patients with AIH type 1 confirming the findings obtained in the paediatric setting and reported that the frequency of CD4^{pos}CD25^{high}FOXP3^{pos} cells is markedly reduced in AIH compared to healthy subjects, that this decrease is more marked when the disease is more active and that the numerical impairment is mirrored by the defective ability of Treg cells to control proliferation of CD4^{pos}CD25^{neg} target cells [4]. We have also shown that the decrease in Tregs is paralleled by a numerical impairment in natural killer T (NKT) cells [4], a subset of cells with regulatory properties, suggesting that, contrary to what proposed by Peiseler *et al.*, a broad immune-regulatory T cell defect is probably present in AIH.

The contrasting peripheral blood results between Peiseler *et al.* and our study probably derive from methodological differences. It is, however, difficult to compare our methods with those used in their paper, as they do not describe in detail their methodology. The purity of their CD4^{pos}CD25^{high} Treg is only 80–90%, and no data are provided to assess the efficiency of the purification protocol they have used (they refer to preliminary results, but do not show them) or whether their purified cells are CD127^{low}. Moreover, in contrast to previous reports [2,3], including ours [4], Peiseler *et al.* use a Treg/T-effector ratio of 1/1 when assessing the suppressor function, while Tregs normally represent some 4% of the total CD4^{+} lymphocyte population in the human blood [5].

Concerning the immunohistochemistry data, Peiseler *et al.* say that their results differ from ours [4], as, in contrast to us, they

find increased numbers of FOXP3^{pos} cells within the inflammatory infiltrate of patients with AIH. In our study, FOXP3^{pos} cells were detected in most liver biopsies from AIH patients, but they represented a small component of the portal tract inflammatory infiltrate. No comparison was made with normal or pathological control livers. As FOXP3^{pos} cells are reportedly absent or scarce in the normal liver, when tested by immunohistochemistry [6,7], it can be argued that we did find increased numbers of tissue FOXP3^{pos} cells in AIH. In support of their findings, Peiseler *et al.* refer to Buckner's review [5], which quotes increased numbers of tissue FOXP3^{pos} cells in human autoimmune disease, such as in the skin in psoriasis and in the gut in inflammatory bowel disease. Interestingly, in the same review [5] Treg function is documented to be decreased in most human autoimmune diseases, including type 1 diabetes, multiple sclerosis, systemic *lupus erythematosus*, rheumatoid arthritis and psoriasis, in agreement with what reported by us [4] and others [2,3] in AIH.

Of note, a numerical defect of peripheral Tregs has been reported in primary biliary cirrhosis (PBC), another autoimmune liver disease [6]. In the same paper, the frequency of FOXP3^{pos} cells within the CD3^{pos} liver infiltrating T lymphocytes was approximately 12% in PBC, 18% in AIH, and 28% in chronic hepatitis C [6], though a more recent paper by Oo *et al.* has reported a frequency of about 12% in both AIH and PBC [7]. As acknowledged by Peiseler *et al.* [1] and Bukner [5], the use of FOXP3 as sole marker of Tregs in tissues is inadequate, FOXP3 being also expressed by effector T cells following activation [5]: more accurate information will be obtained when better markers for immunohistochemical detection of Tregs become available.

Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

References

[1] Peiseler M, Sebode M, Franke B, Wortmann F, Schwinge D, Quaas A, et al. FOXP3+ regulatory T cells in autoimmune hepatitis are fully functional and not reduced in frequency. J Hepatol 2012;57:125–132.
[2] Longhi MS, Ma Y, Bogdanos DP, Cheeseman P, Mieli-Vergani G, Vergani D. Impairment of CD4(+)CD25(+) regulatory T cells in autoimmune liver disease. J Hepatol 2004;41:31–37.
[3] Longhi MS, Hussain MJ, Mitry RR, Arora SK, Mieli-Vergani G, Vergani D, et al. Functional study of CD4+CD25+ regulatory T cells in health and autoimmune hepatitis. J Immunol 2006;176:4484–4491.
[4] Ferri S, Longhi MS, De Molo C, Lalanne C, Muratori P, Granito A, et al. A multifaceted imbalance of T cells with regulatory function characterizes type 1 autoimmune hepatitis. Hepatology 2010;52:999–1007.
[5] Buckner JH. Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases. Nat Rev Immunol 2010;10:849–859.
[6] Lan RY, Cheng C, Lian ZX, Tsuneyama K, Yang GX, Moritoki Y, et al. 6. Liver-targeted and peripheral blood alterations of regulatory T cells in primary biliary cirrhosis. Hepatology 2006;43:729–737.
[7] Oo YH, Weston CJ, Lalor PF, Curbishley SM, Withers DR, Reynolds GM, et al. Distinct roles for CCR4 and CXCR3 in the recruitment and positioning of regulatory T Cells in the inflamed human liver. J Immunol 2010;184:2886–2898.

Silvia Ferri
Claudine Lalanne
Chiara Masi
Luigi Muratori*
Department of Clinical Medicine, University of Bologna, Bologna, Italy
*Corresponding author.
E-mail address: luigi.muratori@unibo.it

Reply to: "Regulatory T cell defects in autoimmune hepatitis"

To the Editor:
We read with interest the comment by Ferri *et al.* to our paper on Tregs in autoimmune hepatitis (AIH) [1], in which they emphasise several discrepancies between their recently published findings [2] and ours. Ferri *et al.* explain these discrepancies by methodological differences; however, find it difficult to compare the methodologies. We are grateful for the opportunity to clarify our methodology in comparison to that used by Ferri *et al.*

(1) Ferri *et al.* report that the frequency of CD4+CD25high-FOXP3+ cells "*is markedly reduced in AIH compared to healthy subjects*", whereas we find that the frequency of CD4+CD25(high)CD127(low)FOXP3+ in blood of AIH patients was not reduced compared to healthy subjects. To determine the Treg frequency in peripheral blood, Ferri *et al.* first purified CD4+CD25+ lymphocytes from peripheral blood by a two-step magnetic purification procedure, i.e. depletion of non-CD4 cells followed by enrichment of CD25+ cells. Only then, the percentage of FOXP3+ cells in the isolated subgroup of blood cells was determined. In contrast, we did not isolate a subfraction of cells from peripheral blood, but instead performed a direct *ex vivo* analysis of unfractionated peripheral blood cells by staining for CD4, CD25, FOXP, and CD127. The gating strategy for this analysis is actually shown in Fig. 1A of our paper. We see at least two critical advantages of our approach: first, by direct *ex vivo* analysis, we avoid the potential selection bias that is inevitable when performing a subfraction analysis after an enrichment procedure; second, by including CD127 as an additional marker, we greatly increase the fidelity of Treg identification. Moreover, we confirmed the findings obtained by this method with another method, i.e. analysis of the *FOXP3* TSDR DNA methylation status.

(2) Ferri *et al.* find a "*defective ability of Treg cells to control the proliferation of CD4+CD25- target cells*"; while we do not find a reduced suppressor function of Treg from AIH patients. To determine Treg suppressor function, Ferri *et al.* used the ^{3}H-thymidine assay; we, in contrast, used