Non-alcoholic steatohepatitis (NASH) and associated end-stage liver disease is a growing cause of concern throughout the Western world. It constitutes a significant clinical burden for which therapeutic approaches are very limited. Over the last years, considerable attention has therefore been paid to identifying potential therapeutic strategies to reduce this burden. Annexin A1 (AnxA1), a calcium-phospholipid binding protein, has been proposed to be a negative regulator of inflammation in the context of NASH. In a recent publication, Gadipudi, Ramavath, Provera et al. investigated the therapeutic potential of Annexin A1 treatment in preventing the progression of NASH. They demonstrate that treatment of mice with NASH with recombinant human AnxA1 can reduce inflammation and fibrosis without affecting steatosis or metabolic syndrome. This was proposed to be achieved through the modulation of the macrophage populations present in the liver. Here, we discuss the main findings of this work and raise some outstanding questions regarding the possible mechanisms involved and the functions of distinct macrophage populations in NASH.

Non-alcoholic fatty liver disease (NAFLD) is quickly becoming one of the most common causes of end-stage liver disease in the Western world [1]. NAFLD represents a spectrum of liver diseases ranging from simple steatosis (lipid accumulation in the liver) to non-alcoholic steatohepatitis (NASH) and NASH-related cirrhosis, which can eventually lead to the development of hepatocellular carcinoma (HCC). With the current lack of effective treatment options, NASH was predicted to become the main cause of liver transplantation by 2030 [2] and indeed just recently NASH was determined to be the fastest growing cause of age-adjusted liver cancer deaths globally [3]. Hepatic inflammation is proposed to be one of the key forces driving the progression from steatosis to NASH and cirrhosis, and interfering with inflammatory pathways has had some success both in pre-clinical and clinical studies [4,5]. In addition, manipulating factors to promote the resolution of inflammation and fibrosis may represent another viable strategy in the treatment of NASH. Annexin A1 (AnxA1) is such a factor that has been shown to promote resolution and impair neutrophil recruitment to sites of inflammation [6]. Moreover, loss of AnxA1 exacerbates metabolic syndrome, hepatic inflammation and fibrosis in different models of NASH [7,8], while its expression in NASH patients is inversely correlated with disease progression [8]. Thus in a recent study, Gadipudi, Ramavath, Provera et al. investigated how treating mice with NASH with recombinant human AnxA1 (hrAnxA1) would influence disease. Interestingly, they found that while this treatment had no effect on metabolic syndrome or hepatic steatosis, it did reduce liver inflammation and fibrosis in two different mouse models of NASH, suggesting this may be a relevant target for therapeutic intervention [9].

In the present study, NASH was first induced by feeding mice either a methionine and choline deficient diet for 4 weeks or a Western diet for 10 weeks and then continuing mice on the diets for an additional 4 or 6 weeks, respectively, in which the mice were treated 5 times per week with hrAnxA1 (intraperitoneal administration). This led to a reduction in the serum alanine aminotransferase levels (sALT) and ameliorated the global histological scoring for lobular inflammation [9]. Furthermore, collagen staining with...
Sirius Red shows a reduction of collagen deposition in mice treated with hrAnxA1, although no changes were detected in terms of steatosis and liver triglyceride content. Similarly, body weight, liver weight and insulin resistance remain unchanged upon hrAnxA1 treatment [9]. In line with reduced inflammation and fibrosis, real-time PCRs (RT-PCRs) on total liver unveiled a reduction in expression of inflammatory markers such as TNFα, CCL2, IL-12p40, CD11b, Pro-Collagen-1α and TGFβ in mice treated with hrAnxA1, suggesting that hrAnxA1 administration is able to reduce hepatic inflammation and fibrosis during NASH progression [9]. However, as these timepoints are still relatively early in the establishment of NASH and fibrosis in these models, it will be interesting to determine if similar results can be obtained when the hrAnxA1 treatment is started later, to better mimic a therapeutic intervention post diagnosis in the clinic.

Within the inflammatory component of NAFLD, hepatic macrophages have been proposed to play key roles in driving the progression to NASH; however, we still understand relatively little regarding the precise roles for these cells. This stems from an underappreciated heterogeneity within the macrophage pool in the liver especially in the setting of liver diseases including NAFLD. This means that for many years, studies have (i) referred to all macrophages in the liver as Kupffer cells (KCs) and (ii) utilized the in vitro M1/M2 macrophage nomenclature to describe ‘KCs’ and this usually based solely on the expression of a couple of marker genes/proteins. First, applying the in vitro M1/M2 nomenclature to infer in vivo macrophage functions based on the expression of a couple of prototypical M1 or M2 genes induces considerable confusion [10]. We have previously shown that homeostatic KCs do not express an overt M1 or M2 signature but rather express a few genes from both sides of the spectrum [11]. Thus, relying on a couple of markers on either side of the spectrum is not sufficient to reach a conclusion regarding an ‘inflammatory M1’ or an ‘anti-inflammatory M2’ phenotype for these cells. Second, recent studies employing state of the art single cell technologies have demonstrated that the hepatic macrophage population in NAFLD consists of multiple populations of macrophages including KCs (in different varieties, see below) and lipid-associated macrophages (LAMs) [12–16], highlighting the need for more specific markers and tools to allow these populations to be discriminated and their specific functions in NASH to be evaluated. With this in mind, KCs can be identified in mice as VSIG4+CLEC2+FOLR2+CLEC4F+ macrophages [12,17] and can be further discriminated into resident KCs which

and their specific functions in NASH to be evaluated. With this in mind, KCs can be identified in mice as VSIG4+CLEC2+FOLR2+CLEC4F+ macrophages [12,17] and can be further discriminated into resident KCs which express TIM4 and CD163 and recently generated monocyte-derived KCs (moKCs) which lack these markers. Moreover, macrophages en route to becoming moKCs in NAFLD can be identified as CLEC2+ before they gain expression of the other KC markers [12–15,18]. Finally, LAMs can be identified through their expression of Gpnmb, Spp1 and Trem2 [12]. Notably, while here we refer to macrophages with this profile and lacking KC markers as LAMs, these cells have been called different names in different studies including NASH-associated macrophages (NAMs) [16] or scar-associated macrophages (SAMs) in the cirrhotic liver [19]. However, as these cells are not specific to the NASH liver [20–23] and do not solely localize around fibrotic scars [17], we prefer the name LAM, which has also been used to describe similar macrophage populations in different tissues and in the homeostatic liver [17,24] and relates to the factors driving, at least part, of their unique signature [17]. Interestingly, in some settings including certain murine models of NASH, KCs can also acquire a LAM-like phenotype, whereby they express markers of both cell types [15,16].

As AnxA1 has been implicated in regulating macrophage functions [8], the authors next sought to examine if hrAnxA1 was exhibiting its effects through modulating these cells. With the aforementioned macrophage heterogeneity in mind, the authors performed RT-qPCRs on total livers of mice fed a WD for 16W and treated with hrAnxA1 during the last 6 weeks of diet. This analysis showed a reduced expression of LAM-specific markers such as Trem2, Spp1 (Osteopentin) and Lgals3 (Galectin 3) and an increased expression of Cd163 [9], a marker of resident KCs [17,18], suggesting a switch in macrophage subsets present in the treated NASH livers. This finding was further corroborated by flow cytometry whereby TIM4+ macrophages were increased and TREM2+ macrophages were decreased, although whether all macrophages are captured in the F4/80+CD11b+ gate used here to define macrophages remains to be seen [9]. Moreover, it is unclear from these data if these populations are mutually exclusive or if the TREM2+ macrophages would also express TIM4 and thus represent LAM-like KCs rather than LAMs. One intriguing finding, however, is that recent transcriptomic profiling of hepatic cells from the healthy and obese murine and human liver [17] has shown that LAMs alongside monocytes, neutrophils and conventional dendritic cells are the main cells expressing Anxa1/ANXA1 (Figure 1, see www.livercellatlas.org). This could thus suggest that the recruitment and differentiation of LAMs may be a self-limiting process, whereby they are recruited, produce AnxA1 and subsequently prevent further generation of LAMs. Notably, this is unlikely to be through an autocrine loop as LAMs do not seem to express the receptor for AnxA1, Formyl peptide receptor 2 (encoded by Fpr2) (Figure 1), suggesting that LAM-derived AnxA1 may act on other cells in the local environment. These findings of a reduced LAM population also correlated with a reduction of macrophages in crown-like structures, a feature of LAMs [12,14]. Taken together these data may suggest that LAMs could be detrimental in NASH, driving inflammation and fibrosis, whereby their loss as mediated
**Figure 1.** Possible mechanisms of action of hrAnxA1 on macrophage populations in NASH

(A) Expression of Anxa1/ANXA1 and Fpr2/FPR2 (mRNA) in all hepatic cells as assessed using single cell and single nuclei RNA sequencing on cells obtained from either the murine (healthy + NASH, where NASH was induced by feeding a Western diet for 24 or 36 weeks) or human (healthy + steatosis, male and female) liver as profiled in [17] and available for interrogation at www.livercellatlas.org.

(B) Schematic representation of different myeloid (macrophage, monocyte and neutrophil) populations in NASH and how treatment with recombinant human Annexin A1 may alter this balance leading to reduced inflammation and fibrosis, while not altering steatosis.

© 2022 The Author(s). This is an open access article published by Portland Press Limited on behalf of the Biochemical Society and distributed under the Creative Commons Attribution License 4.0 (CC BY-NC-ND).
through treatment with hrAnxA1 would confer protection from NASH progression [9]. However, this finding is in direct contrast with a recent study, which demonstrated that a reduction of LAMs in crown-like structures in NASH, mediated by deletion of CCR2, a chemokine receptor needed for monocyte-egress from the bone marrow, worsened fibrosis, suggesting a protective role for LAMs in NASH [14].

How can we explain these discrepancies? While further studies are required to better understand these findings, as the two studies use very different methods that lead to a reduction in LAMs, the obvious explanation is that it is not the loss of LAMs per se that is protective or detrimental but rather the additional effects of these treatments. For example, recent single cell RNA-seq data [17] suggest that the receptor for AnxA1 is expressed on KCs, monocytes and neutrophils in both the mouse and human (Figure 1), thus perhaps hrAnxA1 treatment exerts its effects through these cells, possibly it allows KC numbers to be maintained, which could be achieved through preventing KC death or enabling KC proliferation. Presumably, if KC numbers are not reduced as has been reported across murine models of NASH [12–15,25], then there may be no need to recruit LAMs (Figure 1), although this remains to be tested experimentally. This would be distinct from CCR2 inhibition, which will not affect the KCs, but rather solely alter monocyte recruitment to tissues. Alternatively, another study has suggested that the receptor is expressed by hepatocytes [26], thus perhaps hrAnxA1 treatment acts directly on these cells preventing lipotoxicity and hence the need to generate LAMs. The precise reasons for the differences in terms of expression of the receptor are unclear and hence require further investigation but this may be due to differences between mRNA and protein expression. Moreover, as the expression has been shown to be positively correlated with levels of Estradiol [26], the differences in expression could be due to sex in the RNA-seq study. Another hypothesis regarding the mechanism of action of hrAnxA1 would be that hrAnxA1 treatment specifically interferes with monocyte to LAM differentiation, resulting in a reduced population of LAMs (Figure 1). Alternatively, hrAnxA1 treatment may influence neutrophil recruitment as previously reported [6] and this reduced presence of neutrophils and hence inflammation may prevent KC death and/or LAM recruitment to zones of steatosis (Figure 1). Again, the mechanism of action of CCR2 is distinct preventing/reducing monocyte recruitment to the liver, possibly explaining the difference in phenotype. However, further studies are required to examine these possibilities.

Taken together, the present study has demonstrated that treatment with hrAnxA1 could be a valuable therapeutic strategy to reduce inflammation and fibrosis in the context of NASH. It suggests these beneficial effects are mediated through modulation of the macrophage subsets present which further demonstrates the potential relevance of macrophages in NASH pathogenesis. The present study also further highlights the importance of distinguishing between macrophage subsets when studying the functions of these cells in the context of NASH. The precise mechanisms through which hrAnxA1 treatment modulates the macrophages subsets however, remain to be elucidated, thus, this represents a valuable line of future investigation.

Data Availability
All raw sequencing data and plots to check expression of any gene can be found at www.livercellatlas.org

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

CRediT Author Contribution
Federico F. De Ponti: Visualization, Writing—original draft. Charlotte L. Scott: Conceptualization, Supervision, Funding acquisition, Visualization, Writing—original draft, Writing—review & editing.

Abbreviations
AnxA1, Annexin A1; HCC, hepatocellular carcinoma; KC, Kupffer cell; LAM, lipid-associated macrophage; NAFLD, non-alcoholic fatty liver disease; NAM, NASH-associated macrophage; NASH, non-alcoholic steatohepatitis; RT-PCR, real-time PCR; sALT, serum alanine aminotransferase level; SAM, scar-associated macrophage.

References
1 Eslam, M., Sanyal, A.J. and George, J. (2020) MAFLD: a consensus-driven proposed nomenclature for metabolic associated fatty liver disease. Gastroenterology 158, 1999.e1–2014.e1, https://doi.org/10.1053/j.gastro.2019.11.312
2 Younossi, Z., Anstee, Q.M., Marietti, M., Hardy, T., Henry, L., Eslam, M. et al. (2018) Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. Nat. Rev. Gastroenter. 15, 11–20, https://doi.org/10.1038/nrgastro.2017.109
3 Huang, D.Q., Singal, A.G., Kono, Y., Tan, D.J.H., El-Serag, H.B. and Loomba, R. (2022) Changing global epidemiology of liver cancer from 2010 to 2019: NASH is the fastest growing cause of liver cancer. Cell Metab. 34 (7), 969–977, https://doi.org/10.1016/j.cmet.2022.05.003

© 2022 The Author(s). This is an open access article published by Portland Press Limited on behalf of the Biochemical Society and distributed under the Creative Commons Attribution License 4.0 (CC BY-NC-ND).
4 Reimer, K.C., Wree, A., Roderburg, C. and Tacke, F. (2019) New drugs for NAFLD: lessons from basic models to the clinic. *Hepatol. Int.* 14, 8–23, https://doi.org/10.1007/s12072-019-10001-4

5 Rotman, Y. and Sanyal, A.J. (2016) Current and upcoming pharmacotherapy for non-alcoholic fatty liver disease. *Gut* 66, 180–190, https://doi.org/10.1136/gutjnl-2016-312431

6 Sugimoto, M.A., Vago, J.P., Teixeira, M.M. and Sousa, L.P. (2015) Annexin A1 and the resolution of inflammation: modulation of neutrophil recruitment, apoptosis, and clearance. *J. Immunol. Res.* 2016, 8239258

7 Purvis, G.S.D., Collino, M., Loloi, R.A., Baragetti, A., Chiazza, F., Brovelli, M. et al. (2019) Identification of AnnexinA1 as a novel endogenous regulator of RhoA, and its role in the pathophysiology and experimental therapy of type-2 diabetes. *Front. Immunol.* 10, 571, https://doi.org/10.3389/fimmu.2019.00571

8 Locatelli, I., Sutti, S., Jindal, A., Vacciani, M., Bozzola, C., Reutelingsperger, C. et al. (2014) Endogenous annexin A1 is a novel protective determinant in nonalcoholic steatohepatitis in mice. *Hepatology* 60, 531–544, https://doi.org/10.1002/hep.27141

9 Gadipudi, L.L., Ramavath, N.N., Provera, A., Reutelingsperger, C., Albano, E., Perretti, M. et al. (2022) Annexin A1 treatment prevents the evolution to fibrosis of experimental nonalcoholic steatohepatitis. *Clin. Sci.* 136, 643–656, https://doi.org/10.1042/CS20211122

10 Nahrendorf, M. and Swirski, F.K. (2016) Abandoning M1/M2 for a network model of macrophage function. *Circ. Res.* 119, 414–417, https://doi.org/10.1161/CIRCRESAHA.116.309194

11 Remmerie, A., Martens, L. and Scott, C.L. (2020) Macrophage subsets in obesity, aligning the liver and adipose tissue. *Front. Endocrinol.* 11, 633, https://doi.org/10.3389/fendo.2020.00259

12 Remmerie, A., Martens, L., Thoné, T., Castoldi, A., Seurinck, R., Pavie, B. et al. (2020) Osteopontin expression identifies a subset of recruited macrophages distinct from kupffer cells in the fatty liver. *Immunity* 53, 641.e14–657.e14, https://doi.org/10.1016/j.immuni.2020.08.004

13 Tran, S., Baba, I., Poupel, L., Dussaud, S., Moreau, M., Gélineau, A. et al. (2020) Impaired Kupffer cell self-renewal alters the liver response to lipid overload during non-alcoholic steatohepatitis. *Immunity* 53, 627.e5–640.e5, https://doi.org/10.1016/j.immuni.2020.06.003

14 Daemen, S., Gainullina, A., Kalugotla, G., He, L., Chan, M.M., Beals, J.W. et al. (2021) Dynamic shifts in the composition of resident and recruited macrophages influence tissue remodeling in NASH. *Cell Rep.* 34, 108626, https://doi.org/10.1016/j.celrep.2020.108626

15 Seidman, J.S., Troutman, T.D., Sakai, M., Gola, A., Spann, N.J., Bennett, H. et al. (2020) Niche-specific reprogramming of epigenetic landscapes drives myeloid cell diversity in nonalcoholic steatohepatitis. *Immunity* 52 (6), 1057–1074, https://doi.org/10.1016/j.immuni.2020.04.001

16 Xiong, X., Kuang, H., Ansari, S., Liu, T., Gong, J., Wang, S. et al. (2019) Landscape of intercellular crosstalk in healthy and NASH Liver revealed by single-cell secretome gene analysis. *Mol. Cell.* 75, 644.e5–660.e5, https://doi.org/10.1016/j.molcel.2019.07.028

17 Guilliams, M., Bonnardel, J., Haest, B., Vanderborgh, B., Wagner, C., Remmerie, A. et al. (2022) Spatial proteogenomics reveals distinct and evolutionarily conserved hepatic macrophage niches. *Cell* 185, 379.e38–396.e38, https://doi.org/10.1016/j.cell.2021.12.018

18 Scott, C.L., Zheng, F., Baetselier, P.D., Martens, L., Saëys, Y., Prijck, S.D. et al. (2016) Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells. *Nat. Commun.* 7, 10321, https://doi.org/10.1038/ncomms10321

19 Ramachandran, P., Dobie, R., Wilson-Kanamori, J.R., Dora, E.F., Henderson, B.E.P., Luu, N.T. et al. (2019) Resolving the fibrotic niche of human liver cirrhosis at single-cell level. *Nature* 569, 2–9, https://doi.org/10.1038/s41586-019-1631-3

20 Ben-Moshe, S., Veg, T., Manco, R., Dan, S., Papinutti, D., Lifshitz, A. et al. (2022) The spatiotemporal program of zonal liver regeneration following acute injury. *Nat. Commun.* 7, 973.e10–989.e10, Available from: https://www.nature.com/articles/s41467-020-18973-1, https://doi.org/10.1038/s41467-020-19515-4

21 Kolodziejczyk, A.A., Fedorici, S., Zmora, N., Mohapatra, G., Dori-Bachash, M., Hornstein, S. et al. (2020) Acute liver failure is regulated by MYC- and microbiome-dependent programs. *Nat. Med.* 26, 1899–1911, https://doi.org/10.1038/s41591-020-1102-2

22 Liao, M., Liu, Y., Yuan, J., Wen, Y., Xu, G., Zhao, J. et al. (2020) Single-cell landscape of bronchoalveolar immune cells in patients with COVID-19. *Nat. Med.* 26, 842–844, https://doi.org/10.1038/s41591-020-0901-9

23 Rizzo, G., Vafadarnejad, E., Arampatzi, P., Silvestre, J.S., Zernecke, A., Saliba, A.E. et al. (2020) Single-cell transcriptomic profiling maps monocyte/macrophage transitions after myocardial infarction in mice. *Biorxiv*, 2020.04.14.040451

24 Jahn, D.A., Aduing, L., Thaiss, C.A., Weiner, A., Li, B., Descamps, H. et al. (2019) Lipid-associated macrophages control metabolic homeostasis in a tren2-dependent manner. *Cell* 178, 686.e14–698.e14, https://doi.org/10.1016/j.cell.2019.05.054

25 Devissercher, L., Scott, C.L., Lefere, S., Raevens, S., Bogaerts, E., Paridaens, A. et al. (2017) Non-alcoholic steatohepatitis induces transient changes within the liver macrophage pool. *Cell. Immunol.* 74–83, Available from: http://linkinghub.elsevier.com/retrieve/pii/S0008874917301697, https://doi.org/10.1016/j.cellimm.2017.10.006

26 Lee, C., Kim, J., Han, J., Oh, D., Kim, M., Jeong, H. et al. (2020) Formyl peptide receptor 2 determines sex-specific differences in the progression of nonalcoholic fatty liver disease and steatohepatitis. *Nat. Commun.* 13, 578, https://doi.org/10.1038/s41467-022-28138-6