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Peer Review File

Adipose tissue macrophage infiltration and hepatocyte stress increase GDF-15 throughout development of obesity to MASH

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In the present manuscript, L'homme and colleagues evaluated the potential mechanisms whereby growth differentiation factor-15 (GDF-15) is increased in obesity, type 2 diabetes and metabolic dysfunction-associated liver disease (MASLD) using preclinical models and a large human cohort. The authors found that adipose tissue macrophage infiltration and hepatocyte stress contribute to the increased adipose tissue and liver production of GDF-15 in these metabolic diseases. This is a very interesting, well-designed and timely study shedding light in the underlying mechanism involved in the raise of endogenous GDF-15 levels in metabolic disorders. Nonetheless, some specific points require to be amended.

Specific comments

1. GDF-15 represents one of the most up-regulated proteins during aging and a key molecule in the mechanisms of human stress response (Conte M et al. Aging Res Rev 2022, PMID: 35051643). However, the authors did not find any association of age with the mRNA expression of GDF15 in human SAT, VAT and liver. In addition to age, other factors or confounders such as sex may affect results, which should be adjusted.
2. In the context of obesity and type 2 diabetes, the expression of some hormones is increased, but their receptor signalling is impaired (e.g. leptin, insulin or FGF-21). It would be interesting to characterize the expression of GFRAL, the receptor of GDF-15, in human and murine adipose tissue and liver samples.
3. As regards the expression of GDF15 in adipose tissue macrophages, is it possible to check whether these macrophages have proinflammatory (M1-like) or antiinflamatory (M2-like) phenotype in the conditions studied?

Reviewer #2 (Remarks to the Author):

In this paper, authors address an important question about the GDF15 resource in obesity, type 2 diabetes and MASH. Although it is a very comprehensive study, there are several issues needed to be addressed.
The main point in this paper is that adipose tissue macrophages and hepatocytes are the major resource for the GDF15 levels in MASH/ MASLD, therefore it is very important to test GDF15 levels in mice with depletion of Gdf15 gene in both macrophages and hepatocytes.
What type of macrophages (M1, M2) in AT are important for GDF15 levels in obesity?
Anti-CD115 antibody reduced Gdf15 mRNA expression, what about effect of anti-CD115 antibody on serum GDF15 levels?
To confirm GDF15 is from hepatocytes during MASLD, it is great to have an in vivo experiment about specific deletion of GDF15 in the liver.

For the mechanism of GDF15 secretion, TFEB and DDIT3 are novel factors found in this manuscript. How about GDF15 levels in MASH when deletion of both TFEB and DDIT3 in mice?

Reviewer #3 (Remarks to the Author):

This investigation examined GDF-15 expression and plasma levels that increase with the onset of obesity and T2D and the progression to the liver diseases, MASLD and MASH. Several mouse models of obesity and MASLD as well as biopsies from characterized patients was used in this study. In obesity and T2D the highest levels of GDF-15 were found in the human VAT and the mouse epiAT but not the liver. As the disease progresses to MASLD and MASH the liver became the major source of GDF-15.

The most important finding of the investigation was the identification of the sources of GDF-15. Macrophages that infiltrate into the AT and accumulate with obesity as the source of GDF-15 during obesity and T2D. In contrast the increase in liver GDF-15 is not due to macrophage accumulation but rather due to stress in hepatocytes activating TFEB and DDIT3, two stress-responsive TFs regulating GDF15 expression.

In general, this is a well-done investigation with a very good experimental design. The data thus obtained support the conclusions and the identification of the sources of GDF-15 responsible for the increase in plasma GDF-15 observed during the development of obesity, T2D and MASH. One concern is the poor statistical analysis of the data reported in some of the Figures and Tables and may require further examination by a qualified scientist to determine the validity of the conclusions. To cite one example, see Figure 5 J 4wk and 8wk and Figure 5 G. Analysis at 2wk and 6wk are needed in Figure 5J. In Figure 5G Stats are need to confirm the body weight difference between HFD GDF15 +/- .

Although the data reported in the study point to macrophage infiltration into the AT as being responsible for the increase in plasma GDF-15 in mice on HFD, the more critical complex relationship between macrophage infiltration, GDF-15 and reduction of obesity is not addressed or adequately discussed in the Results and Discussion sections. What is lacking in this investigation is a measurement of the activation of the GFRAL receptor by GDF-15 over the course of the onset of obesity by the HFD. Measurement of food intake or the AT expression of the key enzymes involved in metabolism as reported in several publications are suitable measures of GFRAL-GDF-15 activation. (See Figure5 and related Figures)

Response to Reviewer #1:

General comment: In the present manuscript, L'homme and colleagues evaluated the potential mechanisms whereby growth differentiation factor-15 (GDF-15) is increased in obesity, type 2 diabetes and metabolic dysfunction-associated liver disease (MASLD) using preclinical models and a large human cohort. The authors found that adipose tissue macrophage infiltration and hepatocyte stress contribute to the increased adipose tissue and liver production of GDF-15 in these metabolic diseases. This is a very interesting, well-designed and timely study shedding light in the underlying mechanism involved in the raise of endogenous GDF-15 levels in metabolic disorders. Nonetheless, some specific points require to be amended.

Response to general comment: We thank the reviewer for her/his positive comments and we are pleased that she/he found our work interesting and well-designed.

Comment 1: GDF-15 represents one of the most up-regulated proteins during aging and a key molecule in the mechanisms of human stress response (Conte M et al. Aging Res Rev 2022, PMID: 35051643). However, the authors did not find any association of age with the mRNA expression of GDF15 in human SAT, VAT and liver. In addition to age, other factors or confounders such as sex may affect results, which should be adjusted.

Response 1: We thank the reviewer for bringing up these key points. Indeed, it is largely reported that plasma GDF-15 concentrations strongly associate with age (Bao et al., 2019; Dostálová et al., 2009; Kempf et al., 2007, 2012; Vila et al., 2011; Wiklund et al., 2010). Hence we took the greatest care of matching patients for age and no statistical difference was observed between groups (Supplementary Table 1, 2, 5 and 6). In agreement with the literature cited above, we did observe a correlation between age and plasma GDF-15 levels (Fig. R1). However, and as pointed by the reviewer, we did not observe an association between age and *GDF15* expression in SAT, VAT or liver (Fig. 1F in the manuscript), suggesting that these tissues might not be involved in the elevation of plasma GDF-15 levels with aging.

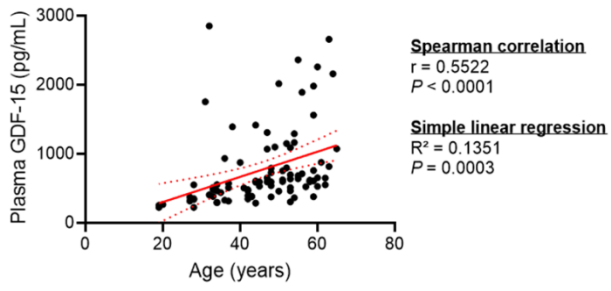

Figure R1. Association of plasma GDF-15 with age.
Correlation between plasma GDF-15 concentrations and age (n = 92, patients described in Supplementary Table 5). Statistical significance was evaluated by the Spearman correlation, a non-parametric measure of rank correlation as used throughout the manuscript for correlations, and by simple linear regression, a parametric method used for analyses below.

Sex is another factor that might impact on circulating GDF-15 concentrations. However, some studies show an association between plasma GDF-15 levels and male attribute (Bao et al., 2019; Kempf et al., 2012) while some other studies show no association with sex (Kempf et al., 2007; Wiklund et al., 2010). In our study, we did not observe a significant association between sex and plasma GDF-15 concentrations (Fig. R2A) or *GDF15* expression in SAT, VAT or liver (Fig. R2B). Figure 1F was updated in the revised manuscript to include sex correlation as illustrated in figure R2B.

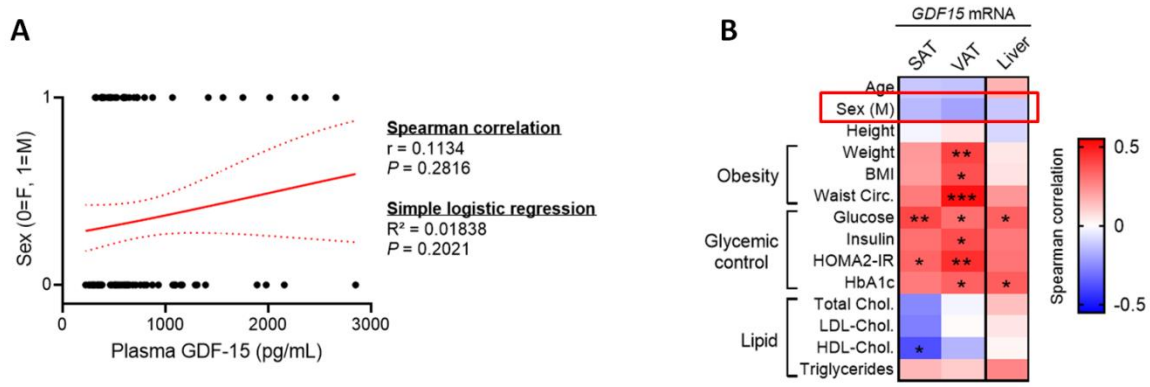

Figure R2. Association of plasma GDF-15 levels and *GDF15* expression with sex.
(A) Correlation between plasma GDF-15 concentrations and sex (n=92, patients described in Supplementary Table 5). Statistical significance was evaluated by the Spearman correlation, a non-parametric measure of rank correlation as used throughout the manuscript for correlations, and by simple logistic regression, a parametric method suitable for binary variable. (**B**) Correlation between GDF15 mRNA expression levels and clinical parameters. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 by Spearman correlation.

To further rule out age or sex as confounders, we performed statistical adjustments as suggested by the reviewer. We first performed simple linear regressions with *GDF15* expression in VAT as dependent variable and clinical parameters as independent variables. With the exception of fasting insulin (*P* = 0.094), all the parameters that we previously identified as positively associated with *GDF15* expression in VAT by Spearman correlations (Fig. 1F in the manuscript) were also positively associated by simple linear regressions (Table R1). After adjustments for age and sex, all these associations remained significant (Table R1, model1), demonstrating that age and sex are not confounders. Additionally, we included BMI in model2 to also adjust for obesity (Table R1). While the associations with other obesity parameters including body weight and waist circumference became not significant as expected, the associations with glycemic parameters remained largely significant after normalization for obesity (Table R1), further supporting our findings that T2D is an additional contributor to GDF-15 production in addition of obesity.

Table R1: Linear regressions for *GDF15* expression in VAT

		Simple regression			Multiple regression (model1)			Multiple regression (model2)		
	N	**Beta**	**95% CI**	***P***	**Beta**	**95% CI**	***P***	**Beta**	**95% CI**	***P***
Age	42	-0.0019	-0.0124, 0.0086	0.713	—	—	—	—	—	—
Sex (M)	42	-0.1092	-0.3343, 0.1159	0.333	—	—	—	—	—	—
BMI	42	**0.0082**	**0.0011, 0.0153**	**0.025**	**0.0081**	**0.0001, 0.0162**	**0.049**	—	—	—

Weight	42	**0.0030**	**0.0005, 0.0056**	**0.022**	**0.0029**	**0.0002, 0.0055**	**0.038**	0.0043	-0.0077, 0.0163	0.474
Waist Circ.	42	**0.0058**	**0.0019, 0.0097**	**0.004**	**0.0057**	**0.0016, 0.0098**	**0.008**	0.0088	-0.0002, 0.0177	0.055
Glucose	42	**0.0468**	**0.0182, 0.0754**	**0.002**	**0.0535**	**0.0247, 0.0822**	**<0.001**	**0.0482**	**0.0172, 0.0792**	**0.003**
Insulin	41	0.0129	-0.0023, 0.0280	0.094	0.0116	-0.0049, 0.0282	0.163	0.0100	-0.0065, 0.0265	0.227
HOMA2-IR	41	**0.1247**	**0.0163, 0.2332**	**0.025**	**0.1200**	**0.0032, 0.2369**	**0.044**	0.1053	-0.0126, 0.2233	0.079
HbA1c	41	**0.0990**	**0.0438, 0.1541**	**<0.001**	**0.1104**	**0.0553, 0.1656**	**<0.001**	**0.1022**	**0.0441, 0.1603**	**0.001**

Simple and multiple linear regressions by using *GDF15* expression in VAT as dependent variable (outcome) and clinical parameters as independent variables (predictors). Model1 was adjusted for age and sex. Model2 was adjusted for age, sex and BMI. Bold values indicate $P < 0.05$. CI, confidence interval.

We also performed similar adjustments for *GDF15* expression in liver. The positive association between *GDF15* expression in liver and liver histology (steatosis, inflammation and ballooning) remained after correction for age and sex (Table R2, model1). Interestingly, further adjustments for obesity and T2D in model2 did not alter the associations with MASH histological parameters (inflammation and ballooning), but abrogated the association with steatosis (Table R2). Together, these new results reinforce our conclusions that obesity, T2D and MASH are three distinct pathophysiological conditions that impact independently and additionally on the expression of *GDF15* in AT and liver. Table R1 and R2 are now included in the revised manuscript as Supplementary Table 3 and 4, and discussed in lines 132-134, 143-145 and 159-161. Methods are provided in lines 1271-1272.

Table R2: Linear regressions for *GDF15* expression in liver

		Simple regression			**Multiple regression (model1)**			**Multiple regression (model2)**		
	N	**Beta**	**95% CI**	***P***	**Beta**	**95% CI**	***P***	**Beta**	**95% CI**	***P***
Age	46	0.0273	-0.0330, 0.0875	0.366	—	—	—	—	—	—
Sex (M)	46	-0.2545	-1.604, 1.095	0.706	—	—	—	—	—	—
BMI	46	0.0348	-0.0225, 0.0921	0.228	0.0373	-0.0210, 0.0955	0.204	—	—	—
HbA1c	43	**0.6283**	**0.1767, 1.080**	**0.008**	**0.6422**	**0.1607, 1.124**	**0.010**	—	—	—
Steatosis	44	**0.7420**	**0.1616, 1.322**	**0.013**	**0.7871**	**0.2019, 1.372**	**0.010**	0.5347	-0.1983, 1.268	0.148
Inflammation	38	**2.113**	**1.233, 2.993**	**<0.001**	**2.105**	**1.179, 3.030**	**<0.001**	**1.946**	**0.8314, 3.060**	**0.001**
Ballooning	38	**1.870**	**1.054, 2.686**	**<0.001**	**1.881**	**1.060, 2.701**	**<0.001**	**1.844**	**0.7788, 2.909**	**0.001**
NAS	38	**0.6049**	**0.3165, 0.8932**	**<0.001**	**0.6178**	**0.3277, 0.9078**	**<0.001**	**0.6003**	**0.2144, 0.9863**	**0.003**
Fibrosis	41	**1.009**	**0.5421, 1.475**	**<0.001**	**0.9951**	**0.5135, 1.477**	**<0.001**	**0.8852**	**0.4012, 1.369**	**<0.001**

Simple and multiple linear regressions by using *GDF15* expression in liver as dependent variable (outcome) and clinical parameters as independent variables (predictors). Model1 was adjusted for age and sex. Model2 was adjusted for age, sex, BMI and HbA1c. Bold values indicate $P < 0.05$. CI, confidence interval.

Comment 2: In the context of obesity and type 2 diabetes, the expression of some hormones is increased, but their receptor signalling is impaired (e.g. leptin, insulin or FGF-21). It would be interesting to characterize the expression of GFRAL, the receptor of GDF-15, in human and murine adipose tissue and liver samples.

Response 2: We appreciate the reviewer comment on this important aspect of GDF-15 function. When GFRAL was discovered as the receptor for GDF-15, its tissue expression was characterized and GFRAL was detected in groups of neurons from the brainstem including the area postrema and the nucleus of the solitary tract (Emmerson et al., 2017; Hsu et al., 2017; Mullican et al., 2017; Yang et al., 2017). Although several studies did not detect GFRAL outside of the brain (Hsu et al., 2017; Igual Gil et al., 2022; Z. Li et al., 2005), there is no consensus on its expression in peripheral tissues and few studies have observed a low expression in certain tissues such as in human adipose tissue (Mullican et al., 2017) or testis (Mullican et al., 2017; Yang et al., 2017). Importantly, *GFRAL* expression in peripheral tissues during metabolic diseases has not been reported so far. As suggested by the reviewer, we have now characterized *GFRAL* expression in mice and humans.

1) In mice, *Gfral* mRNA was scarcely detected in peripheral tissues and only consistently detected in brainstem (Fig. R3A), as previously reported by several groups (Hsu et al., 2017; Igual Gil et al., 2022; Mullican et al., 2017). We did not detect *Gfral* mRNA in adipose tissue or liver and HFD-induced obesity did not modify *Gfral* mRNA detection/expression in any peripheral tissue investigated here. *Gfral* mRNA was detected at low levels in few peripheral tissues, particularly in the small intestine (Fig. R3A). The amplification product obtained in these tissues gave the same melting curve than in the brainstem (Fig. R3B), supporting that the signal corresponds to *Gfral*. However, detection was near the limit of quantification of the qPCR method (Ct > 30-35), likely explaining why we did not detect a signal in every biological replicate. Together, these results confirm previous reports of *Gfral* expression being virtually restricted to the brainstem. Even though *Gfral* is consistently detected in the brainstem, its absolute expression remains low, probably due to its restricted expression in a minority of neurons from the area postrema and the nucleus of the solitary tract (Emmerson et al., 2017; Hsu et al., 2017; Mullican et al., 2017; Yang et al., 2017). It is therefore likely that the low detection in bulk mRNA from peripheral tissues corresponds to a limited number of cells expressing *Gfral*. These cells might be neurons, in line with the detection of *Gfral* expression in brain and small intestine, which contain a complex enteric nervous system.

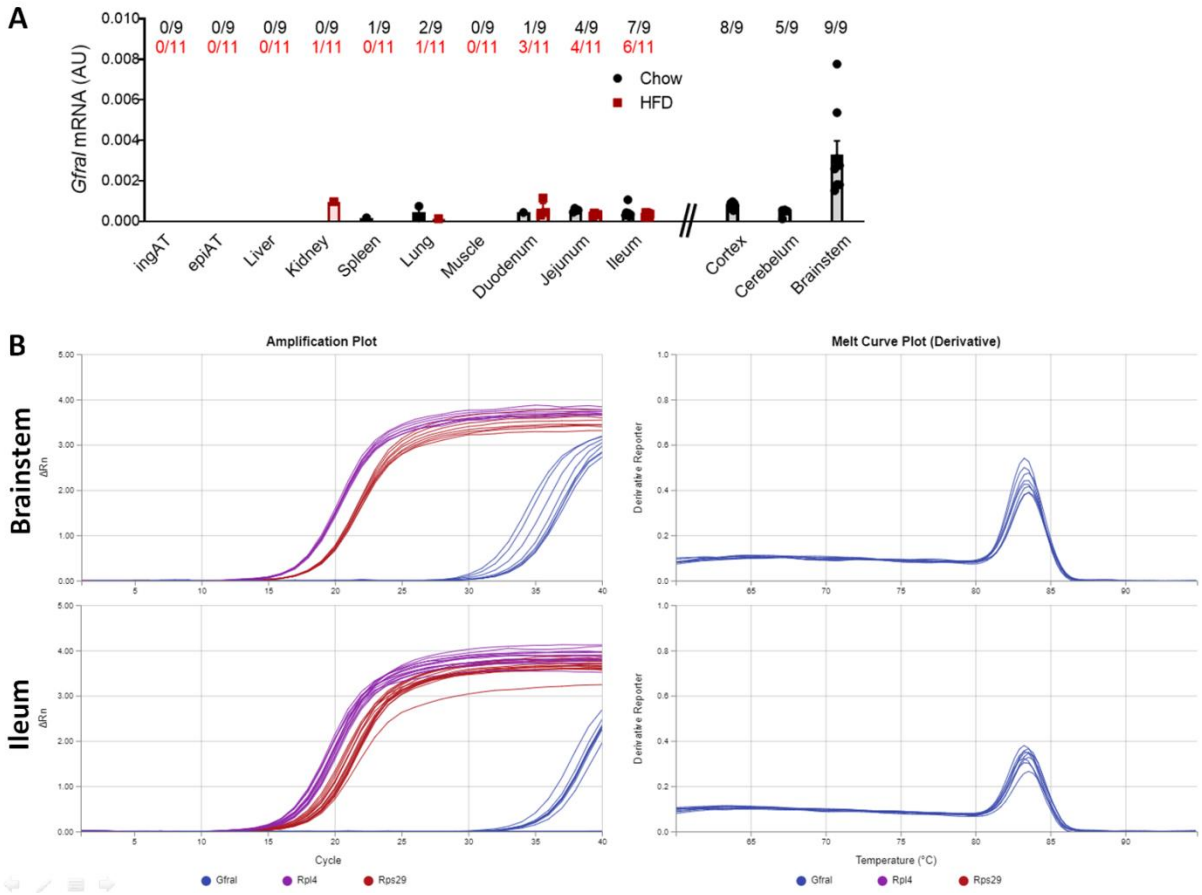

Figure R3. *Gfral* expression in mouse tissues.
(**A**) *Gfral* mRNA expression levels in tissues from mice fed either a chow (n = 9) or a HFD (n = 11) for 12 weeks. Numbers indicate the fraction of mice in which *Gfral* was detected for chow and HFD groups, respectively. Expression in brain (cortex, cerebellum and brainstem) was measured on a separate group of mice. (**B**) Representative amplification and melting curve plots for *Gfral* qPCR (blue) on brainstem (top) and ileum (bottom). Housekeeping genes (*Rpl4* in purple and *Rps29* in red) are also illustrated on the amplification plots. AU, arbitrary unit.

2) In humans, *GFRAL* was detected in adipose tissue (Fig. R4A-B). The expression of *GFRAL* was higher in SAT than VAT, where it was detected in more than 50% of the patients. Identical results were obtained with two different primer pairs. Although detected in most patients, *GFRAL* expression in SAT remains low and near the limit of quantification of the qPCR method (Ct > 30-35). Obesity status did not modify *GFRAL* mRNA detection/expression. As in mice, *GFRAL* was not detected in human liver and the development of liver pathologies, including MASL or MASH, did not result in its detection.

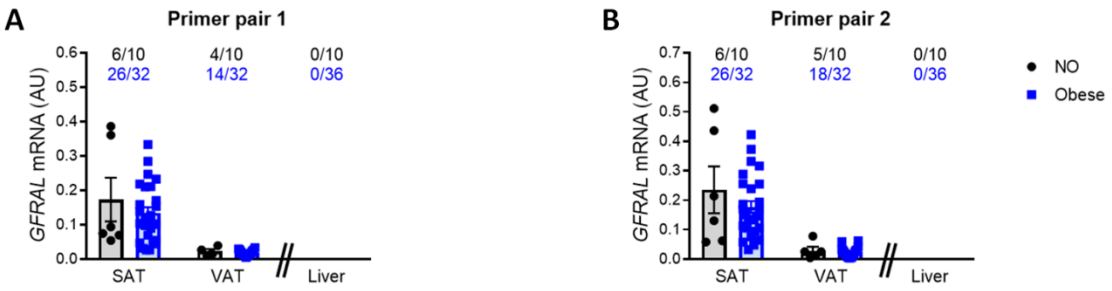

Figure R4. *GFRAL* expression in human AT and liver.
(**A-B**) *GFRAL* mRNA expression levels in paired subcutaneous AT (SAT) and visceral AT (VAT) (n =42) or liver (n = 46) biopsies from patients according to obesity status. Numbers indicate the fraction of patients in which *GFRAL* was detected for Non-Obese (NO) and Obese groups, respectively. AU, arbitrary unit.

The reason for the differential expression of *GFRAL/Gfral* in adipose tissue between mouse and human is unknown. However, it was previously reported that *Gfral* was not detected in adipose tissue from naive mice (Hsu et al., 2017; Igual Gil et al., 2022; Mullican et al., 2017), but was detected in human adipose tissue (Mullican et al., 2017), without further details about the location of adipose tissue and the pathophysiological context of these patients. Here, we showed that SAT expresses more *GFRAL* than VAT and that obesity does not affect *GFRAL* expression/detection.

Together, these results show that *GFRAL* expression is extremely low or undetectable in many tissues. As previously reported (Emmerson et al., 2017; Hsu et al., 2017; Mullican et al., 2017; Yang et al., 2017), the brainstem expresses the highest level of *GFRAL* and is therefore likely the main site of action of GDF-15. However, it cannot be totally excluded that GDF-15 exerts some effects outside of the central nervous system through *GFRAL* expression in the periphery. These new results have been included in the revised manuscript as Supplementary Fig. 1E-G, and discussed in lines 121-122, 127-129 and 469-474. Primer sequences were added in Supplementary Table 7.

Comment 3: As regards the expression of GDF15 in adipose tissue macrophages, is it possible to check whether these macrophages have proinflammatory (M1-like) or antiinflamatory (M2-like) phenotype in the conditions studied?

Response 3: We appreciate the reviewer's insightful comment regarding *GDF15* expression and the macrophage phenotype. To answer this question, we first differentiated mouse bone marrow (BM) cells into BM-derived macrophages (BMDMs) and observed a major upregulation of *Gdf15* expression (Fig. R5A), similar as reported in our manuscript when differentiating mouse monocytes into monocyte-derived macrophages (MDMs) (Fig. 3G in the manuscript). The *in vitro* polarization of BMDMs into M1 macrophages did not modify *Gdf15* expression, while M2 polarization decreased it (Fig. R5B-C). Similarly, M2 polarization of human MDMs decreased *GDF15* expression as well (Fig. R5D-E). Together, these results show that macrophage differentiation considerably increases *Gdf15* expression while further M2 polarization modestly reduces it.

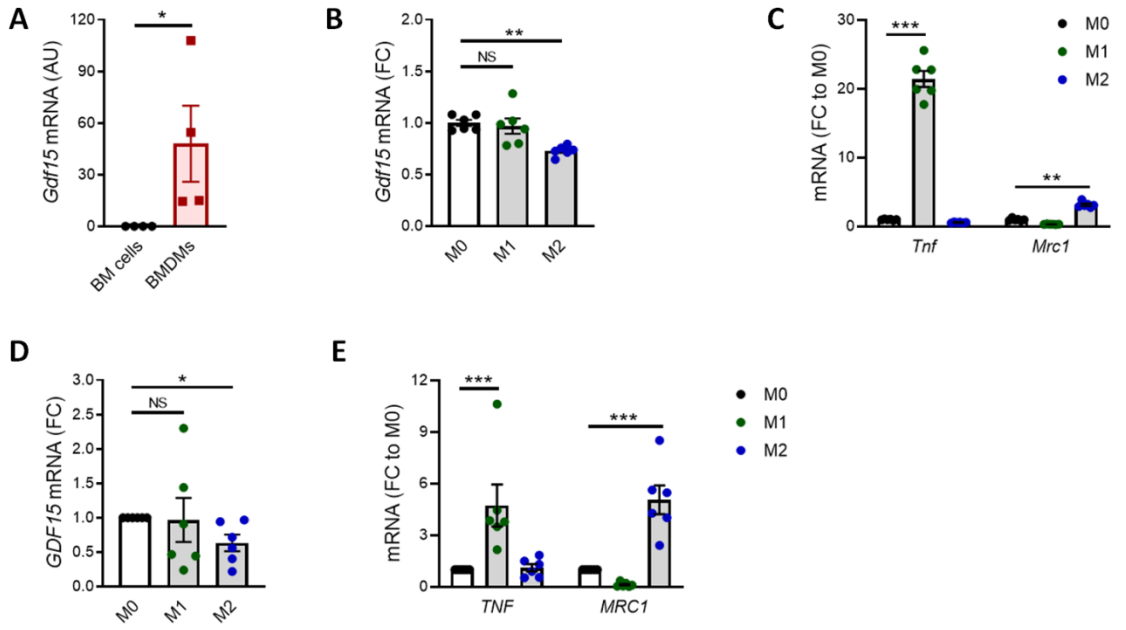

Figure R5. Impact of polarization on GDF15 expression in macrophages.
(A) *Gdf15* mRNA expression levels in freshly isolated bone marrow (BM) cells or in fully differentiated BM-derived macrophages (BMDMs) (n = 4). **(B-C)** *Gdf15, Tnf* or *Mrc1* mRNA expression levels in untreated (M0) or in M1- and M2-polarized BMDMs (n = 6). **(D-E)** *GDF15, TNF* or *MRC1* mRNA expression levels in untreated (M0) or in M1- and M2-polarized human MDMs (n = 6). **P* < 0.05; ***P* < 0.01; ****P* < 0.001 by Mann-Whitney test **(A)**, Kruskal-Wallis test followed by Dunn's multiple comparisons test **(B, D)** or 2-way ANOVA followed by Dunnett's multiple comparisons test **(C, E)**. AU, arbitrary unit; FC, fold change.

Although it was suggested that adipose tissue macrophages (ATMs) may adopt either a M1-like or M2-like polarization profile (Fujisaka et al., 2009; Lumeng et al., 2007), recent studies, including single cell and single nucleus RNA sequencing analyses, challenged the oversimplified M1-M2 model and revealed broader macrophage diversity (Cottam et al., 2022; Hill et al., 2018; Jaitin et al., 2019; C. Li et al., 2019). It has now become well accepted that the previously identified M2-like macrophages corresponds in fact to resident macrophages expressing markers such as CD206 (MRC1), CD163 and LYVE-1 and are predominant in the lean condition, while M1-like macrophages mostly correspond to lipid-associated macrophages (LAMs) expressing TREM2, CD9 and CD11c and are mostly present in obese conditions. We further analyzed the single cell data set used in the manuscript according to this classification and we observed a lower *Gdf15* expression in resident macrophages compared to LAMs or cycling macrophages (Fig. R6A-C), suggesting some heterogeneity in *Gdf15* expression among ATMs. It is interesting to note that both *in vitro* M2-polarized macrophages and epiAT resident macrophages, that share several phenotypical characteristics, express lower levels of *Gdf15*.

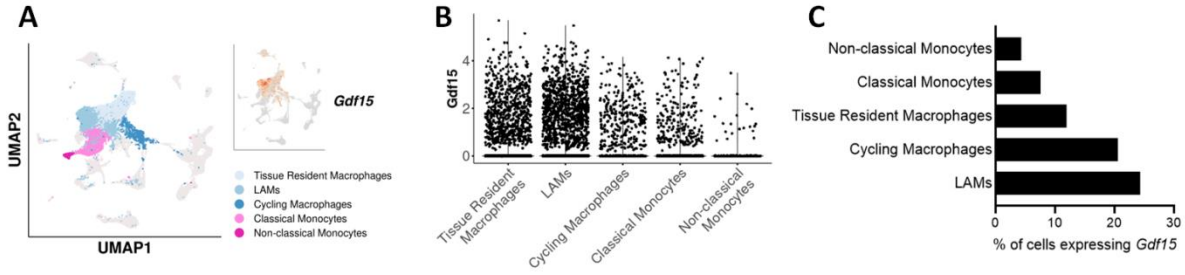

Figure R6. *Gdf15* expression in monocytes and macrophages clusters from epiAT.
(A-C) Monocytes/macrophages clusters and *Gdf15* mRNA expression levels visualized in a UMAP representation **(A)** or as a violin plot with data points **(B)** from scRNA-seq of CD45+ cells from epiAT of mice on HFD feeding (33,322 single cells). **(C)** Percentage of cells expressing *Gdf15* according to monocytes/macrophages cluster.

Although *Gdf15* expression displays variation among ATM subpopulations and the ATM subpopulations ratio changes with obesity, we did not observe differences in *Gdf15* expression in the whole ATMs population between mice fed a chow or HFD for 12 weeks (Fig. 3F in the manuscript). However, we demonstrated that the amount of macrophages is directly related to the expression of *Gdf15* in epiAT (Fig. 3 to 5 in the manuscript). It is therefore likely that macrophage recruitment/differentiation is the most important determinant of *GDF15* expression in AT during obesity while the impact of macrophage polarization/subtype is of minor importance. We now included these results in the revised manuscript (Supplementary Fig. 3D-F, Supplementary Fig. 3N-P and Supplementary Fig. 4I-J) and discussed them in lines 234-236, 274-277, 294-296 and 483-488. Methods are provided in lines 1033-1041. Primer sequences were added in Supplementary Table 7.

References related to Reviewer #1:

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Response to Reviewer #2:

General comment: In this paper, authors address an important question about the GDF15 resource in obesity, type 2 diabetes and MASH. Although it is a very comprehensive study, there are several issues needed to be addressed.

Response to general comment: We thank the reviewer for the insightful comments and we are glad that she/he recognizes the importance of the question addressed by our work.

Comment 1: The main point in this paper is that adipose tissue macrophages and hepatocytes are the major resource for the GDF15 levels in MASH/ MASLD, therefore it is very important to test GDF15 levels in mice with depletion of Gdf15 gene in both macrophages and hepatocytes.

Response 1: We appreciate the reviewer comment about the dual contribution of macrophages and hepatocytes which is indeed a key point in our manuscript. In fact, only hepatocytes are a source of GDF-15 in MASH/MASLD. Indeed, macrophage depletion had no impact on *Gdf15* expression in mouse liver (Fig. 6A-B in the manuscript), and macrophage content is not associated with the expression of *GDF15* in human liver (Fig. 6C in the manuscript). However, macrophages are a source of GDF-15 in obesity and T2D. Since obesity and MASH/MASLD are frequently associated, both macrophages and hepatocytes can contribute to increased GDF-15 levels.

The creation of a mouse line with *Gdf15* inactivation in both macrophages and hepatocytes, for instance by crossing *Gdf15*$^{fl/fl}$ mice with Alb-Cre and LysM-Cre mice, might be of interest to assess the extent of the combined contribution of macrophages and hepatocytes on GDF-15 levels in a model of obesity with MASH. However, in addition to the substantial time required to generate such a mouse line (more than a year), this model is not suitable to accurately determine the respective contribution of macrophages or hepatocytes in a specific pathophysiological context (obesity or MASH). While we demonstrated the contribution of macrophages in obesity using 3 different *in vivo* models (macrophage depletion with anti-CD115, siGdf15-GeRPs and BMT, see figure 5 in the manuscript), we did not demonstrate the contribution of hepatocytes to GDF-15 production in MASH *in vivo*.

Thus, as also requested by the reviewer in comment 4, we now demonstrate the role of hepatocytes on the increase of *Gdf15* expression in liver and plasma GDF-15 concentrations in MASH *in vivo*. For this purpose, we fed mice a CDAA diet for 4 weeks to induce an obesity-independent MASH-like condition and we delivered siRNA targeting *Gdf15* four days prior sacrifice by using invivofectamine 3.0 reagent, lipid-based nanoparticles providing high efficiency delivery of siRNA to hepatocytes following tail vein injection (Wrobel et al., 2021). As expected, the injection of siRNA targeting *Gdf15* decreased *Gdf15* expression in liver (Fig. R7A), but not in other tissues expressing high levels of *Gdf15* including adipose tissue and kidney (Fig. R7B). Plasma GDF-15 concentrations were also reduced (Fig. R7C), while no difference was observed prior siRNA injections (Fig. R7D). Interestingly, CDAA-fed mice injected with siRNA targeting *Gdf15* gained slightly more weight than control mice (Fig. R7E-F), suggesting that CDAA-induced weight loss is at least partially due to the increase of plasma GDF-15 concentrations following its upregulated expression by

hepatocytes. We have now included these results in the revised manuscript (Fig. 6F-G & Supplementary Fig. 8F-I) and discussed them in lines 374-382. Methods are provided in lines 1008-1020.

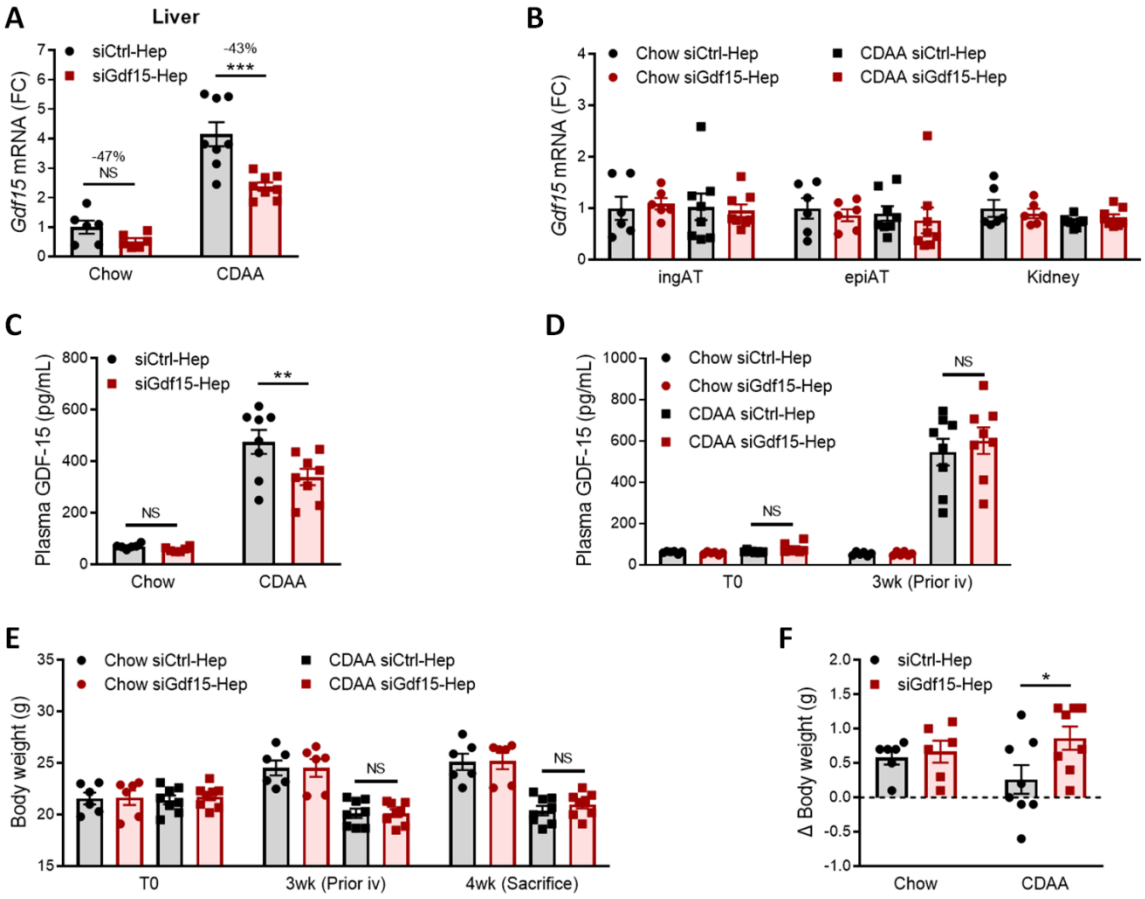


Figure R7. Inactivation of *Gdf15* in hepatocytes.
(A-F) Mice were fed a chow or CDAA diet for 4 weeks and tail vein injected with invivofectamine-siRNA complex (1.5 mg/kg siRNA) 4 days prior sacrifice (n = 6-8/group). **(A)** *Gdf15* mRNA expression levels in liver. **(B)** *Gdf15* mRNA expression levels in adipose tissues and kidney. **(C)** Plasma GDF-15 concentrations at sacrifice (4 weeks). **(D)** Plasma GDF-15 concentrations prior diet intervention (T0) and prior tail vein injections (3 weeks on diet). **(E)** Evolution of body weight. **(F)** Body weight gain during the week of injection (body weight 4th week minus 3rd week). Data are shown as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001 by 2-way ANOVA followed by Sidak's multiple comparisons test (**A**, **C**, **F**) or Holm-Sidak's multiple comparisons test (**E**) . FC, fold change; NS, not significant.

Comment 2: What type of macrophages (M1, M2) in AT are important for GDF15 levels in obesity?

Response 2: We appreciate the reviewer's insightful comment regarding *GDF15* expression and the macrophage phenotype. To answer this question, we first differentiated mouse bone marrow (BM) cells into BM-derived macrophages (BMDMs) and observed a major upregulation of *Gdf15* expression (Fig. R8A), similar as reported in our manuscript when differentiating mouse monocytes into monocyte-derived macrophages (MDMs) (Fig. 3G in the manuscript). The *in vitro* polarization of BMDMs into M1 macrophages did not modify

Gdf15 expression, while M2 polarization decreased it (Fig. R8B-C). Similarly, M2 polarization of human MDMs decreased *GDF15* expression as well (Fig. R8D-E). Together, these results show that macrophage differentiation considerably increases *Gdf15* expression while further M2 polarization modestly reduces it.

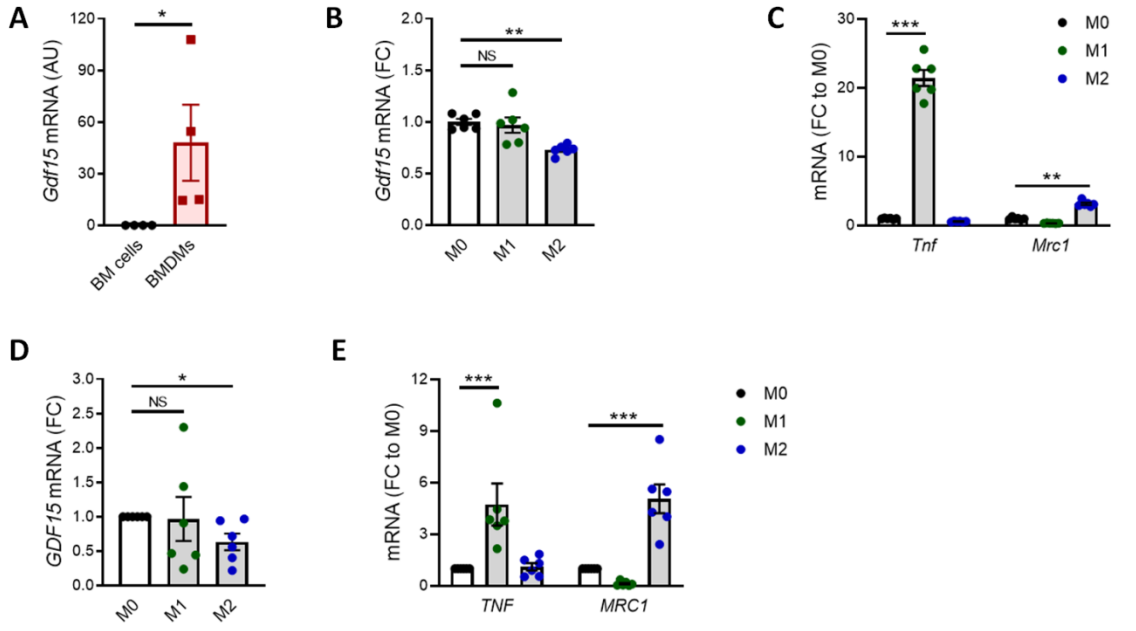


Figure R8. Impact of polarization on GDF15 expression in macrophages.
(A) *Gdf15* mRNA expression levels in freshly isolated bone marrow (BM) cells or in fully differentiated BM-derived macrophages (BMDMs) (n = 4). **(B-C)** *Gdf15, Tnf* or *Mrc1* mRNA expression levels in untreated (M0) or in M1- and M2-polarized BMDMs (n = 6). **(D-E)** *GDF15, TNF* or *MRC1* mRNA expression levels in untreated (M0) or in M1- and M2-polarized human MDMs (n = 6). **P* < 0.05; ***P* < 0.01; ****P* < 0.001 by Mann-Whitney test **(A)**, Kruskal-Wallis test followed by Dunn's multiple comparisons test **(B, D)** or 2-way ANOVA followed by Dunnett's multiple comparisons test **(C, E)**. AU, arbitrary unit; FC, fold change.

Although it was suggested that adipose tissue macrophages (ATMs) may adopt either a M1-like or M2-like polarization profile (Fujisaka et al., 2009; Lumeng et al., 2007), recent studies, including single cell and single nucleus RNA sequencing analyses, challenged the oversimplified M1-M2 model and revealed broader macrophage diversity (Cottam et al., 2022; Hill et al., 2018; Jaitin et al., 2019; C. Li et al., 2019). It has now become well accepted that the previously identified M2-like macrophages corresponds in fact to resident macrophages expressing markers such as CD206 (MRC1), CD163 and LYVE-1 and are predominant in the lean condition, while M1-like macrophages mostly correspond to lipid-associated macrophages (LAMs) expressing TREM2, CD9 and CD11c and are mostly present in obese conditions. We further analyzed the single cell data set used in the manuscript according to this classification and we observed a lower *Gdf15* expression in resident macrophages compared to LAMs or cycling macrophages (Fig. R9A-C), suggesting some heterogeneity in *Gdf15* expression among ATMs. It is interesting to note that both *in vitro* M2-polarized macrophages and epiAT resident macrophages, that share several phenotypical characteristics, express lower levels of *Gdf15*.

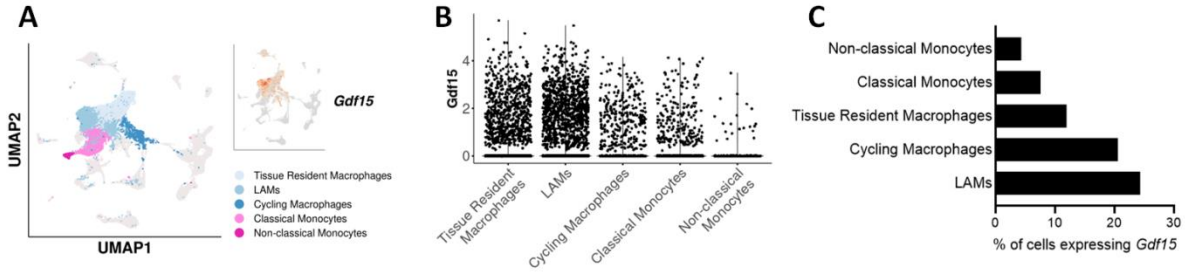


Figure R9. *Gdf15* expression in monocytes and macrophages clusters from epiAT.
(A-C) Monocytes/macrophages clusters and *Gdf15* mRNA expression levels visualized in a UMAP representation **(A)** or as a violin plot with data points **(B)** from scRNA-seq of CD45+ cells from epiAT of mice on HFD feeding (33,322 single cells). **(C)** Percentage of cells expressing *Gdf15* according to monocytes/macrophages cluster.

Although *Gdf15* expression displays variation among ATM subpopulations and the ATM subpopulations ratio changes with obesity, we did not observe differences in *Gdf15* expression in the whole ATMs population between mice fed a chow or HFD for 12 weeks (Fig. 3F in the manuscript). However, we demonstrated that the amount of macrophages is directly related to the expression of *Gdf15* in epiAT (Fig. 3 to 5 in the manuscript). It is therefore likely that macrophage recruitment/differentiation is the most important determinant of *GDF15* expression in AT during obesity while the impact of macrophage polarization/subtype is of minor importance. We now included these results in the revised manuscript (Supplementary Fig. 3D-F, Supplementary Fig. 3N-P and Supplementary Fig. 4I-J) and discussed them in lines 234-236, 274-277, 294-296 and 483-488. Methods are provided in lines 1033-1041. Primer sequences were added in Supplementary Table 7.

Comment 3: *Anti-CD115 antibody reduced Gdf15 mRNA expression, what about effect of anti-CD115 antibody on serum GDF15 levels?*

Response 3: We did measure plasma GDF-15 concentrations after macrophage depletion by acute injection of anti-CD115 antibody and we did not observe a decline in plasma GDF-15 concentrations (Fig. R10A). Surprisingly, plasma GDF-15 level increased, mostly in the chow group, while no difference was observed before randomization and injection (Fig. R10B). This unexpected observation led us to consider that some macrophages may encounter uncontrolled cell death and partial release of their cellular content, including GDF-15 which is highly expressed by macrophages. Supporting this hypothesis, we did observe a general increase of plasma HMGB1 levels (Fig. R10C), a protein released after various types of cell death (Chen et al., 2022). This may be explained by the fact that antibody-mediated cell depletion involves several immune processes such as the antibody-dependent cellular phagocytosis (ADCP), which minimize the release of cellular content from targeted cells, but also involves antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) that both lead to the release of cellular components upon death (Carter, 2006).

To further support the involvement of macrophage cell death on the increased plasma GDF-15 concentrations during macrophage depletion, we also injected chow-fed mice with clodronate liposomes which deplete macrophages by specifically inducing their death (Van Rooijen & Sanders, 1994). Four days after a single clodronate liposome injection (same timing as in the

macrophage depletion experiment with the anti-CD115 antibody), macrophage depletion was achieved and confirmed by qPCR for *Adgre1* (F4/80) expression in epiAT (Fig. R10D). In an even more striking way than with anti-CD115 injection, mice injected with clodronate liposomes displayed a robust increase of plasma GDF-15 and HMGB1 levels (Fig. R10E-F), suggesting that macrophage death during macrophage depletion may favor the release of their intracellular GDF-15. Thus, to demonstrate the contribution of macrophages to plasma GDF-15 levels during obesity, we used two different models directly targeting *Gdf15* expression in macrophages, si*Gdf15*-GeRPs and BMT from *Gdf15*$^{-/-}$ mice, and showed that the inactivation of *Gdf15* in macrophages reduced plasma GDF-15 concentrations (Fig 5E and 5J in the manuscript).

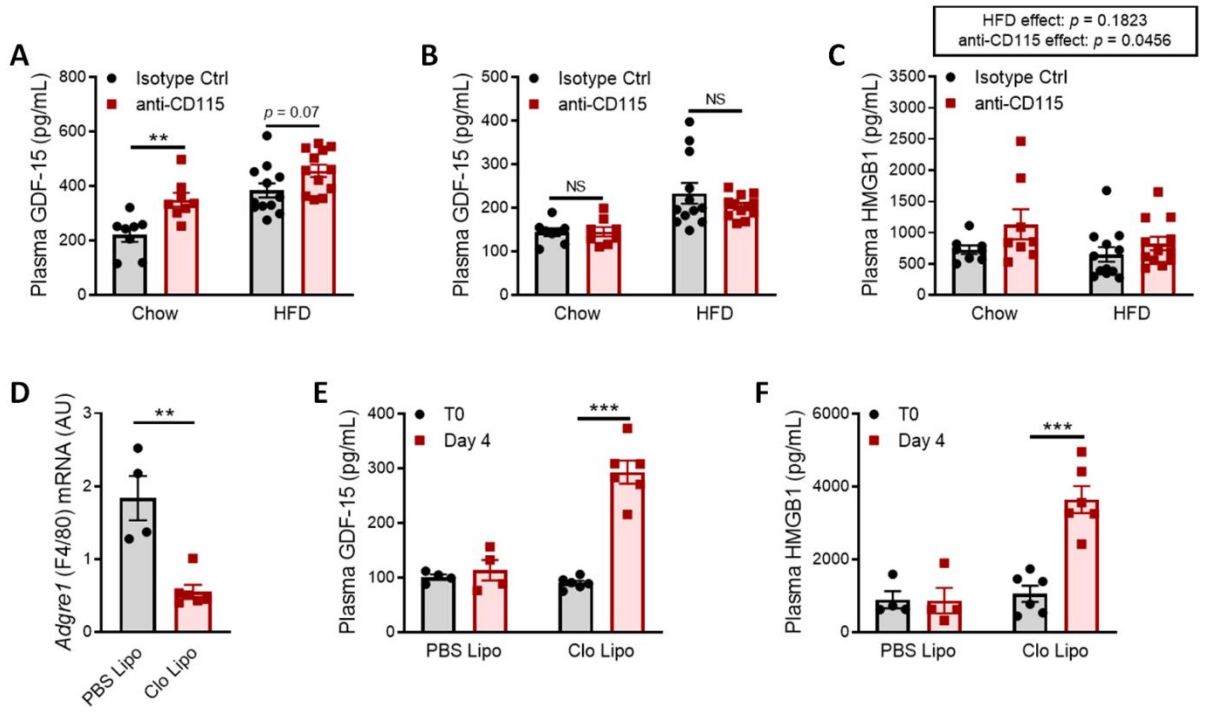


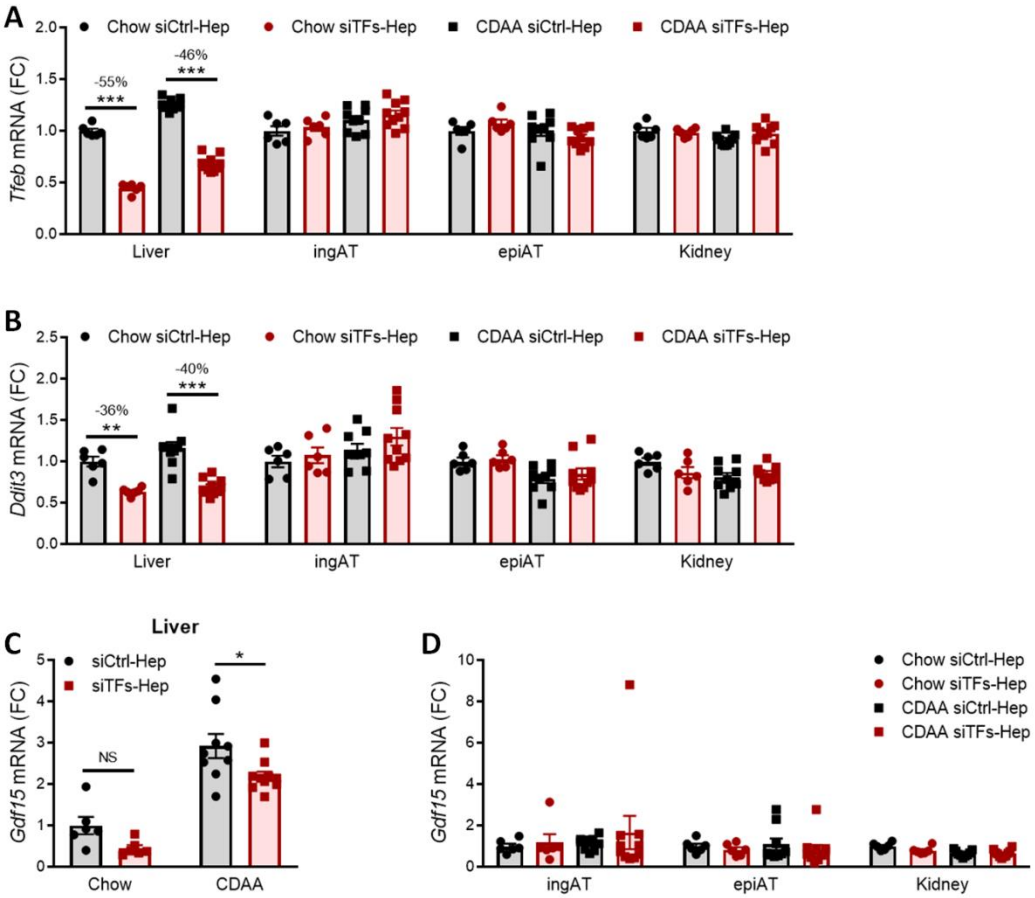
Figure R10. Impact of macrophage deletion on plasma GDF-15 concentrations.
(A-C) Mice were fed a chow or a HFD for 12 weeks followed by a single intraperitoneal injection of anti-CD115 antibody or isotype control (n = 8-12/group). Mice were sacrificed after 4 days. **(A)** Plasma GDF-15 concentrations at sacrifice. **(B)** Plasma GDF-15 concentrations after 12 weeks of HFD and prior intraperitoneal injections. **(C)** Plasma HMGB1 concentrations at sacrifice measured by ELISA (Novus Biologicals cat#NBP2-62767). **(D-F)** Chow-fed mice were intraperitoneally injected with 10 ml/kg of ready-to-use clodronate liposomes or PBS liposomes (LIPOSOMA research liposomes cat#CP-010-010) for 4 days. **(D)** *Gdf15* mRNA expression levels in epiAT. **(E)** Plasma GDF-15 concentrations prior intraperitoneal injections (T0) and at sacrifice (Day 4). **(F)** Plasma HMGB1 concentrations prior intraperitoneal injections (T0) and at sacrifice (Day 4). Data are shown as mean ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 by 2-way ANOVA followed by Sidak's multiple comparisons test **(A**, **B**, **C, E, F)** or Mann-Whitney test **(D)**. AU, arbitrary unit; NS, not significant.

Comment 4: To confirm GDF15 is from hepatocytes during MASLD, it is great to have an in vivo experiment about specific deletion of GDF15 in the liver.

Response 4: As further detailed above in response 1, we have now performed this *in vivo* experiment and demonstrated the role of hepatocytes on the elevation of *Gdf15* expression in liver and plasma GDF-15 concentrations during MASLD.

Comment 5: For the mechanism of GDF15 secretion, TFEB and DDIT3 are novel factors found in this manuscript. How about GDF15 levels in MASH when deletion of both TFEB and DDIT3 in mice?

Response 5: We sincerely appreciate the reviewer constructive comment. As performed for GDF-15 and detailed in response 1, we have now inactivated the two transcription factors (TFs) TFEB and DDIT3 in hepatocytes by using invivofectamine-siRNA complexes. Injection of siRNA targeting *Tfeb* and *Ddit3* (siTFs-Hep) decreased the expression of both genes in liver, but not in adipose tissue or kidney (Fig. R11A-B). The inactivation of the two TFs also decreased *Gdf15* expression in liver and plasma GDF-15 concentrations in CDAA-fed mice (Fig. R11C & R11E). We observed no difference in *Gdf15* expression in other tissues expressing high levels of *Gdf15*, including adipose tissue and kidney (Fig. R11D). Prior to siRNA injections, plasma GDF-15 concentrations were similar between groups (Fig. R11F). CDAA-fed mice injected with siRNA targeting the two TFs gained slightly more weight than control mice but, unlike for *Gdf15* silencing, this did not reach statistical significance (Fig. R11G-H). This difference between TFs and *Gdf15* silencing likely results from their respective ability to decrease plasma GDF-15 concentrations. TFs inactivation has a lower impact on plasma GDF-15 levels than the direct silencing of *Gdf15* and possibly requires a longer time to affect body weight. Altogether these results confirm our *in vitro* findings showing that TFEB and DDIT3 regulate *GDF15* expression in hepatocytes and demonstrate the involvement of these two TFs in hepatocyte-derived GDF-15 production during MASH development *in vivo*. We have now included these results in the revised manuscript (Fig. 6L-M & Supplementary Fig. 11A-F) and discussed them in lines 446-451 and 539-541.



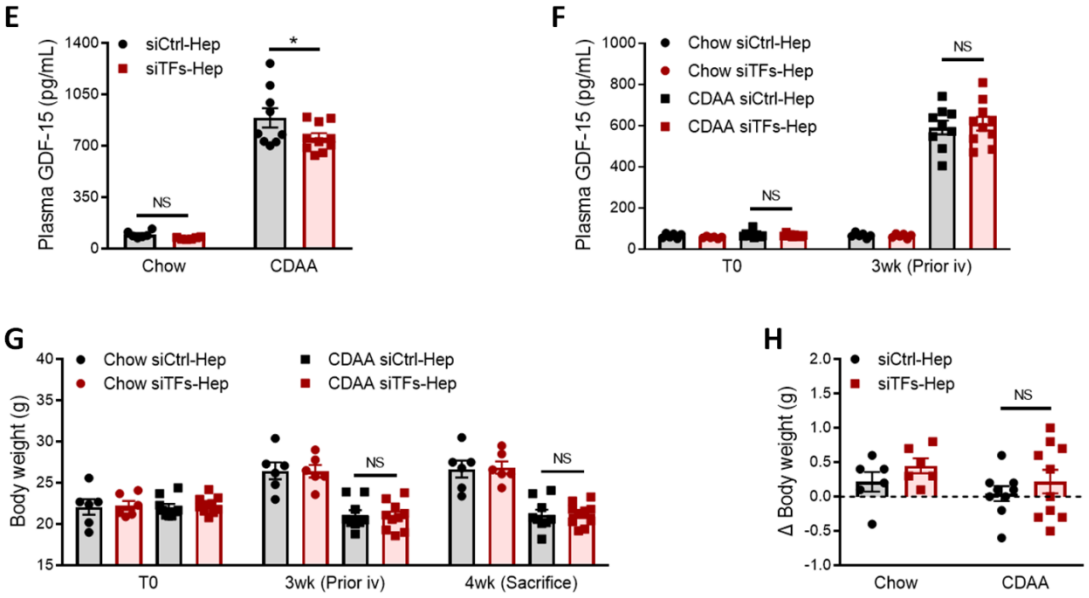


Figure R11. Inactivation of *Tfeb and Ddit3* in hepatocytes.
(A-H) Mice were fed a chow or CDAA diet for 4 weeks and tail vein injected with invivofectamine-siRNA complex (3 mg/kg siRNA) 4 days prior sacrifice (n = 6-10/group). **(A)** *Tfeb* mRNA expression levels in liver, adipose tissues and kidney. **(B)** *Ddit3* mRNA expression levels in liver, adipose tissues and kidney. **(C)** *Gdf15* mRNA expression levels in liver. **(D)** *Gdf15* mRNA expression levels in adipose tissues and kidney. **(E)** Plasma GDF-15 concentrations at sacrifice (4 weeks). **(F)** Plasma GDF-15 concentrations prior diet intervention (T0) and prior tail vein injections (3 weeks on diet). **(G)** Evolution of body weight. **(H)** Body weight gain during the week of injection (body weight 4th week minus 3rd week). Data are shown as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001 by 2-way ANOVA followed by Holm-Sidak's multiple comparisons test (**A, B, G)** or by Sidak's multiple comparisons test (**C, E, F, H)**. FC, fold change; NS, not significant.

References related to Reviewer #2:

Carter, P. J. (2006). Potent antibody therapeutics by design. *Nature Reviews. Immunology*, *6*(5), 343–357. https://doi.org/10.1038/nri1837

Chen, R., Kang, R., & Tang, D. (2022). The mechanism of HMGB1 secretion and release. *Experimental & Molecular Medicine*, *54*(2), 91–102. https://doi.org/10.1038/s12276-022-00736-w

Van Rooijen, N., & Sanders, A. (1994). Liposome mediated depletion of macrophages: Mechanism of action, preparation of liposomes and applications. *Journal of Immunological Methods*, *174*(1–2), 83–93. https://doi.org/10.1016/0022-1759(94)90012-4

Wrobel, L., Siddiqi, F. H., & Rubinsztein, D. C. (2021). Transient siRNA-mediated protein knockdown in mouse followed by feeding/starving cycle and liver tissue analysis. *STAR Protocols*, *2*(2), 100500. https://doi.org/10.1016/j.xpro.2021.100500

Response to Reviewer #3:

General comment: This investigation examined GDF-15 expression and plasma levels that increase with the onset of obesity and T2D and the progression to the liver diseases, MASLD and MASH. Several mouse models of obesity and MASLD as well as biopsies from characterized patients was used in this study. In obesity and T2D the highest levels of GDF-15 were found in the human VAT and the mouse epiAT but not the liver. As the disease progresses to MASLD and MASH the liver became the major source of GDF-15.

The most important finding of the investigation was the identification of the sources of GDF-15. Macrophages that infiltrate into the AT and accumulate with obesity as the source of GDF-15 during obesity and T2D. In contrast the increase in liver GDF-15 is not due to macrophage accumulation but rather due to stress in hepatocytes activating TFEB and DDIT3, two stress-responsive TFs regulating GDF15 expression.

In general, this is a well-done investigation with a very good experimental design. The data thus obtained support the conclusions and the identification of the sources of GDF-15 responsible for the increase in plasma GDF-15 observed during the development of obesity, T2D and MASH.

Response to general comment: We thank the reviewer for her/his positive comments and we are pleased that she/he found our work well-designed and our conclusions supported by the data.

Comment 1: One concern is the poor statistical analysis of the data reported in some of the Figures and Tables and may require further examination by a qualified scientist to determine the validity of the conclusions. To cite one example, see Figure 5 J 4wk and 8wk and Figure 5 G.

Response 1: The statistical analysis was reexamined as requested by reviewer. We noticed that some statistical information was indeed missing such as the mention that tests were two-tailed, the alpha level selected, the reason of the use of non-parametric tests, statistics on figure 5G or the use of Dunnett's correction. These pieces of information have now been included in the revised manuscript (lines 1264-1270 and 1281).

We however did not find flaws in our initial statistical analyses. Our manuscript complies with most renowned statistical recommendations, such as the SAMPL (Statistical Analyses and Methods in the Published Literature) guidelines (Lang & Altman, 2015), and with the *Nature Communications* guidelines. A comprehensive description of the statistical analyses is provided and, as strongly encouraged by *Nature Communications*, we have now provided raw data (Source Data), allowing any reader to replicate or challenge our statistical analyses.

We took the greatest care to select the most appropriated statistical test and we systematically corrected for multiple comparisons. We chose to mostly use non-parametric tests for several reasons. For human data, we noticed that plasma GDF-15 levels and GDF15 expression displayed an asymmetry of distribution, characterized by several extremely high values in obese patients while levels in non-obese patients seemed normally distributed (Fig. R12A-B). Histogram representation confirmed that data have a right skewed distribution and density

curve in blue largely deviate from normal distribution calculated from data (red) (Fig. R12C-D). Moreover, the Shapiro-Wilk normality test showed that data significantly deviate from a normal distribution, leading us to consider GDF-15 as non-normally distributed in our cohort. For the *in vitro* and mice experiments, whenever possible, we also performed non-parametric tests as sample sizes were too small to guarantee data normality. As is well known, non-parametric tests may have lower statistical power than parametric tests for very small sample sizes (Fig. R12E). Although we are fully aware that such approach might increase type II error ("false negative", $P > 0.05$ while there is a biological effect), it however tends to restrain type I error ("false positive", $P < 0.05$ while there is no biological effect) and therefore strengthen conclusions when stating about the significance of results. When two independent variables were considered, such as diet and genotype, we used the parametric test 2-way ANOVA as there is no proper non-parametric alternative and statistical software such as GraphPad Prism has no other solution.

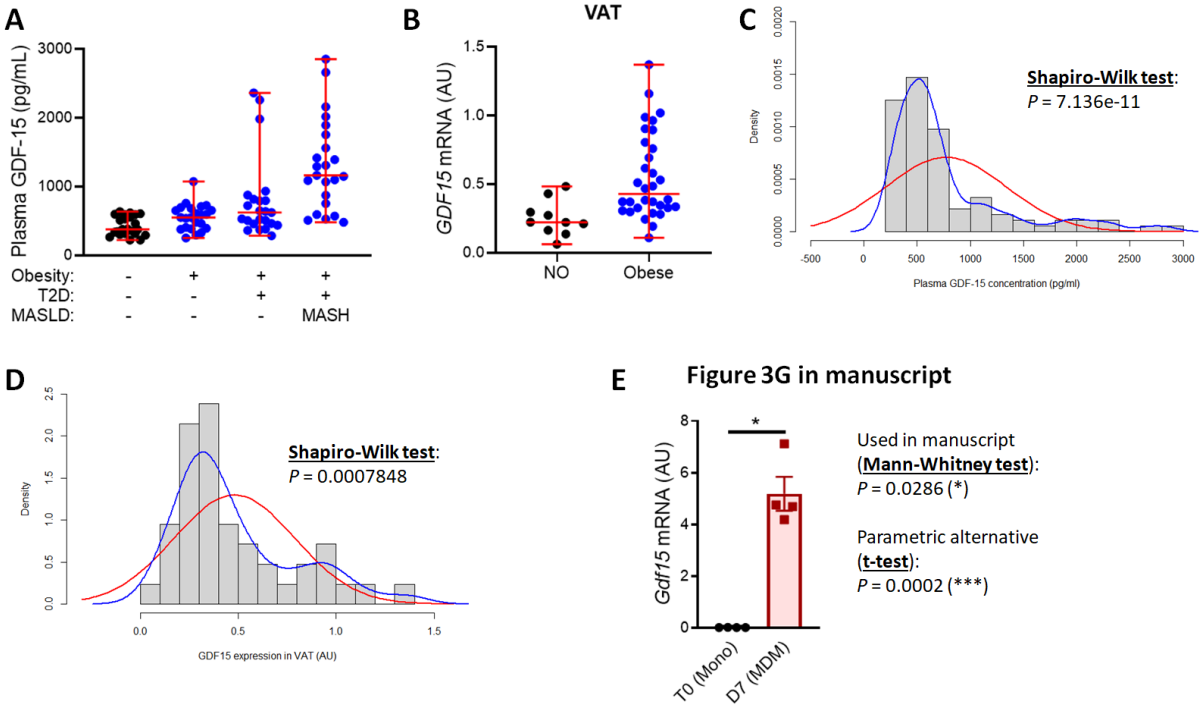


Figure R12. Distribution of plasma GDF-15 concentrations and *GDF15* expression levels.
(A) Plasma GDF-15 concentrations in patient according to obesity, T2D and MASLD adapted from figure 2E in the manuscript (n = 23/group). Red lines represent median with range (min to max). **(B)** *GDF15* mRNA expression levels in human VAT adapted from figure 1D in the manuscript (n = 42). Red lines represent median with range (min to max). **(C-D)** Histogram of plasma GDF-15 concentration (n = 92) **(C)** and *GDF15* mRNA expression levels in human VAT (n = 42) **(D)**. Density curve is represented in blue and calculated normal curve according to data in red. **(E)** Figure 3G from the manuscript representing *Gdf15* mRNA expression levels in freshly sorted monocytes (T0) or in fully differentiated MDM after 7 days (D7). Both Mann-Whitney and t-test were performed. AU, arbitrary unit; NO, Non-Obese.

The reviewer points out two examples of "poor statistics", figure 5J and figure 5G. In figure 5J, we performed 2-way ANOVA followed by Holm-Sidak's multiple comparisons test. Figure R13 shows adjusted *P* values obtained with GraphPad Prism and reported on the graph. As detailed earlier, we systematically corrected for multiple comparisons to minimize

type I errors. Holm-Sidak's correction is a powerful method preferred over too conservative methods such as Bonferroni, which gives in the current example (4 weeks) an adjusted *P* value of 0.0636 (instead of 0.0212 with Holm-Sidak's correction). Another robust correction is by controlling the false discovery rate (FDR) by using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Adjusted q values obtained with this robust method were very similar to Holm-Sidak's correction and confirmed the significance of our result at 4 weeks (*q* value of 0.0222) (Fig. R14).

ANOVA results | Multiple comparisons

2way ANOVA
Multiple comparisons

4	Number of comparisons per family	3			
5	Alpha	0.05			
6					
7	**Holm-Šídák's multiple comparisons test**	**Mean Diff.**	**Below threshold?**	**Summary**	**Adjusted P Value**
8					
9	T0				
10	HFD BMT *Gdf15*$^{+/+}$ vs. Chow BMT *Gdf15*$^{+/+}$	-2.902	No	ns	0.9167
11	HFD BMT *Gdf15*$^{+/+}$ vs. Chow BMT *Gdf15*$^{-/-}$	35.88	No	ns	0.5382
12	HFD BMT *Gdf15*$^{+/+}$ vs. HFD BMT *Gdf15*$^{-/-}$	32.84	No	ns	0.5382
13					
14	4wk				
15	HFD BMT *Gdf15*$^{+/+}$ vs. Chow BMT *Gdf15*$^{+/+}$	112.7	Yes	**	0.0026
16	HFD BMT *Gdf15*$^{+/+}$ vs. Chow BMT *Gdf15*$^{-/-}$	161.0	Yes	****	<0.0001
17	HFD BMT *Gdf15*$^{+/+}$ vs. HFD BMT *Gdf15*$^{-/-}$	65.96	Yes	*	0.0212
18					
19	8wk				
20	HFD BMT *Gdf15*$^{+/+}$ vs. Chow BMT *Gdf15*$^{+/+}$	146.2	Yes	**	0.0064
21	HFD BMT *Gdf15*$^{+/+}$ vs. Chow BMT *Gdf15*$^{-/-}$	190.1	Yes	**	0.0013
22	HFD BMT *Gdf15*$^{+/+}$ vs. HFD BMT *Gdf15*$^{-/-}$	45.69	No	ns	0.2877
23					
24	12wk				
25	HFD BMT *Gdf15*$^{+/+}$ vs. Chow BMT *Gdf15*$^{+/+}$	219.6	Yes	****	<0.0001
26	HFD BMT *Gdf15*$^{+/+}$ vs. Chow BMT *Gdf15*$^{-/-}$	237.8	Yes	****	<0.0001
27	HFD BMT *Gdf15*$^{+/+}$ vs. HFD BMT *Gdf15*$^{-/-}$	-43.99	No	ns	0.3891

Figure R13. Adjusted *P* values from Holm-Sidak's multiple comparisons.
2-way ANOVA followed by Holm-Sidak's multiple comparisons test was performed with GraphPad Prism on data represented in figure 5J in the manuscript.

ANOVA results | Multiple comparisons

	2way ANOVA Multiple comparisons				
4	Number of comparisons per family	3			
5	Q	0.05			
6					
7	**Two-stage linear step-up procedure of Benjamini, Krieger and Yekutiel**	**Mean Diff.**	**Discovery?**	**q value**	**Individual P Value**
8					
9	T0				
10	HFD BMT *Gdf15*$^{+/+}$ vs. Chow BMT *Gdf15*$^{+/+}$	-2.902	No	0.9626	0.9167
11	HFD BMT *Gdf15*$^{+/+}$ vs. Chow BMT *Gdf15*$^{-/-}$	35.88	No	0.4856	0.3083
12	HFD BMT *Gdf15*$^{+/+}$ vs. HFD BMT *Gdf15*$^{-/-}$	32.84	No	0.4856	0.2271
13					
14	4wk				
15	HFD BMT *Gdf15*$^{+/+}$ vs. Chow BMT *Gdf15*$^{+/+}$	112.7	Yes	0.0021	0.0013
16	HFD BMT *Gdf15*$^{+/+}$ vs. Chow BMT *Gdf15*$^{-/-}$	161.0	Yes	<0.0001	<0.0001
17	HFD BMT *Gdf15*$^{+/+}$ vs. HFD BMT *Gdf15*$^{-/-}$	65.96	Yes	0.0222	0.0212
18					
19	8wk				
20	HFD BMT *Gdf15*$^{+/+}$ vs. Chow BMT *Gdf15*$^{+/+}$	146.2	Yes	0.0017	0.0032
21	HFD BMT *Gdf15*$^{+/+}$ vs. Chow BMT *Gdf15*$^{-/-}$	190.1	Yes	0.0005	0.0004
22	HFD BMT *Gdf15*$^{+/+}$ vs. HFD BMT *Gdf15*$^{-/-}$	45.69	No	0.1007	0.2877
23					
24	12wk				
25	HFD BMT *Gdf15*$^{+/+}$ vs. Chow BMT *Gdf15*$^{+/+}$	219.6	Yes	<0.0001	<0.0001
26	HFD BMT *Gdf15*$^{+/+}$ vs. Chow BMT *Gdf15*$^{-/-}$	237.8	Yes	<0.0001	<0.0001
27	HFD BMT *Gdf15*$^{+/+}$ vs. HFD BMT *Gdf15*$^{-/-}$	-43.99	No	0.1362	0.3891

Figure R14. q values from two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.
2-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli was performed with GraphPad Prism on data represented in figure 5J in the manuscript.

The second and last example concerns figure 5G from which statistical analysis was omitted. We greatly thank the reviewer for pointing out this omission. We now performed 2-way ANOVA and reported the statistical results (Fig. R15). As we concluded in the manuscript, *Gdf15*$^{-/-}$ BMT mice gained more weight than *Gdf15*$^{+/+}$ BMT mice on HFD feeding (genotype effect $P = 0.0053$) but not on chow diet feeding (genotype effect $P = 0.2639$). Figure 5G has now been updated as illustrated in figure R15.

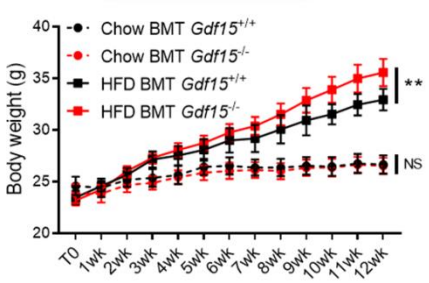


Figure R15. Evolution of body weight.
After bone marrow transplantation (BMT) from Gdf15$^{+/+}$ or Gdf15$^{-/-}$ mice, reconstituted mice were fed a chow or a HFD for 12 weeks (n = 5-11/group). Body weight was measured weekly. $^{**}P < 0.01$ by 2-way ANOVA for the factor genotype (Gdf15$^{+/+}$ or Gdf15$^{-/-}$). NS, not significant.

Comment 2: Analysis at 2wk and 6wk are needed in Figure 5J.

Response 2: Unfortunately, blood was not collected at 2 and 6 weeks in the BMT experiment depicted in figure 5J, but only at T0 and after 4, 8 and 12 weeks of HFD. For ethical reasons, we limited blood collection to minimize the stress incurred by the animals and to favor optimal body weight gain. This was crucial in this experiment as BMT is well known to promote resistance to HFD-induced obesity (Katiraei et al., 2017). We considered that collecting blood every 4 weeks allowed us to follow the progressive increase of GDF-15 concentrations as observed in a preliminary experiment (Fig. R16).

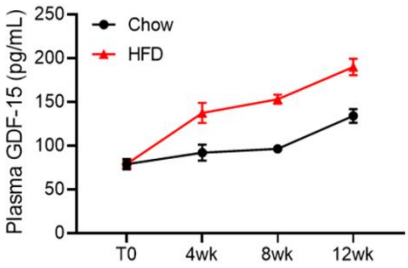


Figure R16. Evolution of plasma GDF-15 concentrations during HFD-induced obesity.
C57BL/6J mice were fed a chow or HFD for 12 weeks and blood was collected from tail every 4 weeks to measure plasma GDF-15 concentrations by ELISA (n = 6-9/group).

Comment 3: In Figure 5G Stats are need to confirm the body weight difference between HFD GDF15 +/- .

Response 3: We thank once again the reviewer for bringing this omission to our attention. As explained in response 1, statistical analysis was performed and the figure was updated accordingly. As we concluded in the manuscript, the difference in body weight gain was significant.

Comment 4: Although the data reported in the study point to macrophage infiltration into the AT as being responsible for the increase in plasma GDF-15 in mice on HFD, the more critical complex relationship between macrophage infiltration, GDF-15 and reduction of obesity is not addressed or adequately discussed in the Results and Discussion sections. What is lacking in this investigation is a measurement of the activation of the GFRAL receptor by GDF-15 over the course of the onset of obesity by the HFD. Measurement of food intake or the AT expression of the key enzymes involved in metabolism as reported in several publications are suitable measures of GFRAL-GDF-15 activation. (See Figure5 and related Figures)

Response 4: We highly appreciate the reviewer's critical comments about GFRAL activation. GFRAL is the only known receptor for GDF-15 which is mostly expressed by brainstem neurons in the area postrema and in the nucleus of the solitary tract (Emmerson et al., 2017; Hsu et al., 2017; Mullican et al., 2017; Yang et al., 2017). GDF15-related GFRAL activation in these brain structures induces two main effects, a reduction in food intake (Emmerson et al., 2017; Hsu et al., 2017; Mullican et al., 2017; Yang et al., 2017) and the activation of β-

adrenergic signaling in the periphery (Sjøberg et al., 2023; Wang et al., 2023), both contributing to weight loss and obesity protection.

We did measure the food intake at the end of the bone marrow transplantation (BMT) experiment. Mice, which were co-housed for the ten first weeks on diet, were single housed to measure food intake until sacrifice at 12 weeks. We observed that cumulative food intake was higher in *Gdf15*$^{-/-}$ BMT mice than *Gdf15*$^{+/+}$ BMT mice on HFD feeding (Fig. R17). Unfortunately, food intake was not performed over the entire course of HFD-induced obesity to limit the total duration of isolation-induced animal stress. We have now included these results in the revised manuscript (Supplementary Fig. 7J-K) and discussed them in lines 339-340. Methods are provided in lines 1003-1007.

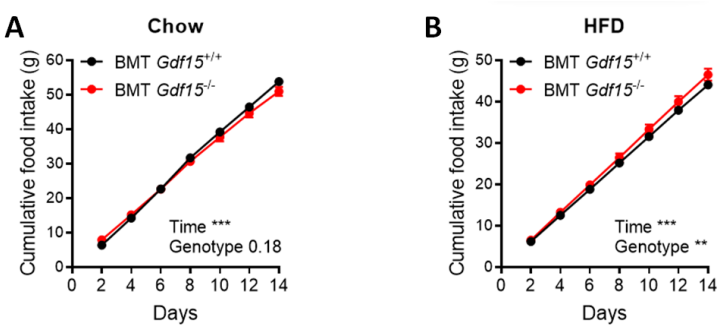


Figure R17. Food intake.
(A-B) After bone marrow transplantation (BMT) from *Gdf15*$^{+/+}$ or *Gdf15*$^{-/-}$ mice, reconstituted mice were co-housed at 2/2 ratio (BMT *Gdf15*$^{+/+}$/BMT *Gdf15*$^{-/-}$) and fed a chow or a HFD for 10 weeks. Two weeks prior sacrifice, mice were single housed to measure food intake. **(A)** Food intake in chow-fed mice (n = 5/group). **(B)** Food intake in HFD-fed mice (n = 11/group).

The second major effect of GFRAL activation is the induction of the sympathetic system and β-adrenergic signaling in peripheral tissues that increases energy expenditure (Wang et al., 2023) and insulin sensitivity (Sjøberg et al., 2023). Overexpression or injection of recombinant GDF-15 have also been shown to increase thermogenesis in BAT (Chrysovergis et al., 2014) and the expression of genes involved in lipolysis, lipogenesis and fatty acid oxidation in liver and adipose tissue (Chrysovergis et al., 2014; Chung et al., 2017), likely through the β-adrenergic system. However, whether endogenous GDF-15 has similar effects is unclear. As requested by the reviewer, we analyzed the expression of key enzymes involved in lipid metabolism in epiAT and liver (Fig. R18), including the genes previously shown to be increased by overexpression or recombinant GDF-15 (Chrysovergis et al., 2014; Chung et al., 2017). However, we did not observe major differences between the genotypes. Importantly, it should be highlighted that plasma GDF-15 concentrations were no longer different at the time of sacrifice (Fig. 5J in the manuscript), likely explaining the lack of effect at this time-point.

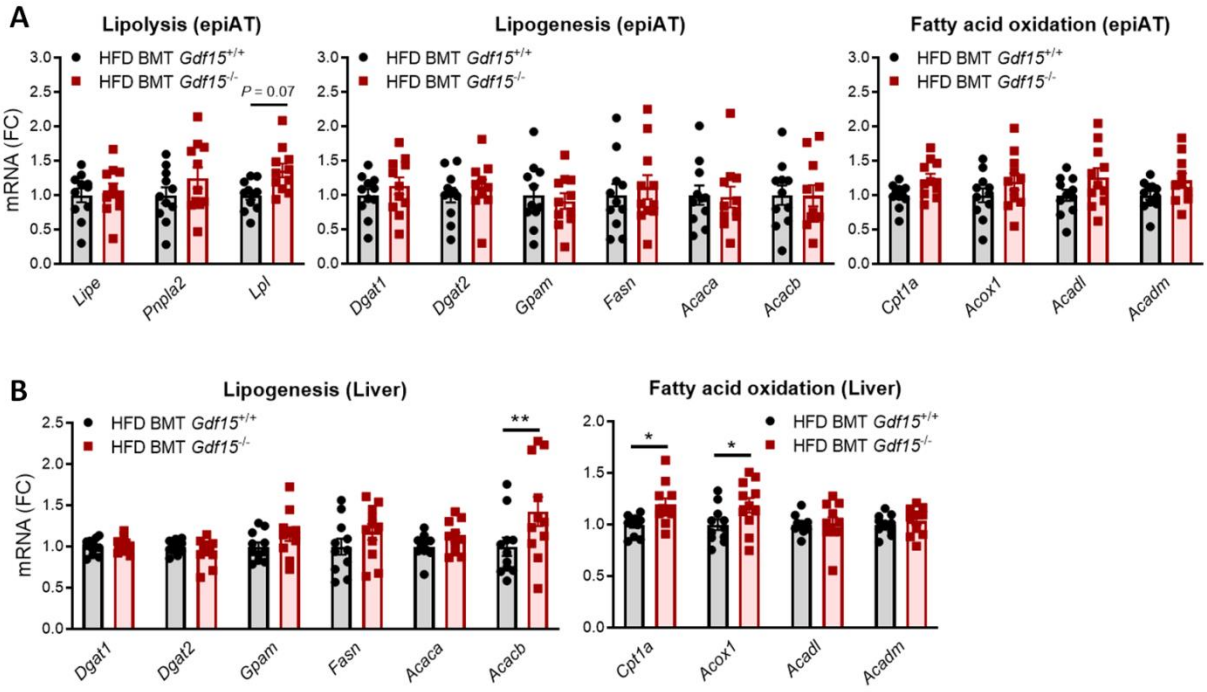


Figure R18. Expression of genes involved in lipid metabolism.
(A-B) After bone marrow transplantation (BMT) from Gdf15$^{+/+}$ or Gdf15$^{-/-}$ mice, reconstituted mice were fed a HFD for 12 weeks (n = 11/group). mRNA expression levels of key enzymes involved in lipid metabolism in epiAT **(A)** and liver **(B)**. *P < 0.05; **P < 0.01 by 2-way ANOVA followed by Sidak's multiple comparisons test **(A, B)**. FC, fold change.

GFRAL activation is the only widely accepted mechanism of action of GDF-15 (Emmerson et al., 2017; Hsu et al., 2017; Mullican et al., 2017; Sjøberg et al., 2023; Wang et al., 2023; Yang et al., 2017). As it was already extensively reported that GDF-15 protects against obesity through GFRAL, we focused our study on the identification of the source of GDF-15 in metabolic diseases and the determination of the molecular mechanisms regulating its expression. We have now integrated these considerations about GFRAL in the discussion (lines 469-474).

References related to Reviewer #3:

Chrysovergis, K., Wang, X., Kosak, J., Lee, S.-H., Kim, J. S., Foley, J. F., Travlos, G., Singh, S., Baek, S. J., & Eling, T. E. (2014). NAG-1/GDF-15 prevents obesity by increasing thermogenesis, lipolysis and oxidative metabolism. *International Journal of Obesity (2005)*, *38*(12), 1555–1564. https://doi.org/10.1038/ijo.2014.27

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J., Barnard, G. C., Tang, J. X., Liu, X., Mao, X., Siegel, R., Sloan, J. H., … Wu, X. (2017). The metabolic effects of GDF15 are mediated by the orphan receptor GFRAL. *Nature Medicine*, *23*(10), 1215–1219. https://doi.org/10.1038/nm.4393

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Mullican, S. E., Lin-Schmidt, X., Chin, C.-N., Chavez, J. A., Furman, J. L., Armstrong, A. A., Beck, S. C., South, V. J., Dinh, T. Q., Cash-Mason, T. D., Cavanaugh, C. R., Nelson, S., Huang, C., Hunter, M. J., & Rangwala, S. M. (2017). GFRAL is the receptor for GDF15 and the ligand promotes weight loss in mice and nonhuman primates. *Nature Medicine*, *23*(10), 1150–1157. https://doi.org/10.1038/nm.4392

Sjøberg, K. A., Sigvardsen, C. M., Alvarado-Diaz, A., Andersen, N. R., Larance, M., Seeley, R. J., Schjerling, P., Knudsen, J. G., Katzilieris-Petras, G., Clemmensen, C., Jørgensen, S. B., De Bock, K., & Richter, E. A. (2023). GDF15 increases insulin action in the liver and adipose tissue via a β-adrenergic receptor-mediated mechanism. *Cell Metabolism*, *35*(8), 1327-1340.e5. https://doi.org/10.1016/j.cmet.2023.06.016

Wang, D., Townsend, L. K., DesOrmeaux, G. J., Frangos, S. M., Batchuluun, B., Dumont, L., Kuhre, R. E., Ahmadi, E., Hu, S., Rebalka, I. A., Gautam, J., Jabile, M. J. T., Pileggi, C. A., Rehal, S., Desjardins, E. M., Tsakiridis, E. E., Lally, J. S. V., Juracic, E. S., Tupling, A. R., … Steinberg, G. R. (2023). GDF15 promotes weight loss by enhancing energy expenditure in muscle. *Nature*, *619*(7968), 143–150. https://doi.org/10.1038/s41586-023-06249-4

Yang, L., Chang, C.-C., Sun, Z., Madsen, D., Zhu, H., Padkjær, S. B., Wu, X., Huang, T., Hultman, K., Paulsen, S. J., Wang, J., Bugge, A., Frantzen, J. B., Nørgaard, P., Jeppesen, J. F., Yang, Z., Secher, A., Chen, H., Li, X., … Jørgensen, S. B. (2017). GFRAL is the receptor for GDF15 and is required for the anti-obesity effects of the ligand. *Nature Medicine*, *23*(10), 1158–1166. https://doi.org/10.1038/nm.4394

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

I thank the Authors for addressing all my comments/suggestions. The manuscript has been substantially improved after revision and it is my belief that it is now ready for publication in Nature Communications.

Reviewer #2 (Remarks to the Author):

The injection of siRNA targeting Gdf15 in hepatocytes are not very convincing. MASH model still has very higher GDF15 gene level in liver and protein level in blood compared to chow diet, which suggests that some other organs or other cell types in the liver contribute to higher GDF15 in MASH. So what are they?

I like the response in my comment 2.

Inactivation of Gdf15 in macrophages in BMT model did not reduce plasma GDF-15 concentrations in later phase in fig. 5J. What are the possible reasons?

What about food intake data for the models injected with siRNA targeting Tfeb and Ddit3?

Reviewer #3 (Remarks to the Author):

Adequately addressed the comments of the reviewers.

Response to Reviewer #2:

Comment 1: The injection of siRNA targeting Gdf15 in hepatocytes are not very convincing. MASH model still has very higher GDF15 gene level in liver and protein level in blood compared to chow diet, which suggests that some other organs or other cell types in the liver contribute to higher GDF15 in MASH. So what are they?

Response 1: We do agree with reviewer that the injection of siRNA targeting *Gdf15* in hepatocytes did not fully prevent CDAA-induced *Gdf15* expression in liver or plasma GDF-15 concentrations (Fig. 6F-G in the manuscript). However, this is highly likely due to intrinsic technical problems with siRNA-silencing rather than to an extra-hepatic source of GDF-15 (see below).

Liver is the key source of GDF-15: We believe that our data convincingly support the conclusion that liver is the major, if not the only, source of GDF-15 in mice fed a CDAA diet. The CDAA diet primarily impacts the liver[1,2] and we observed an increase of *Gdf15* expression only in liver, but not in ingAT, epiAT and kidney (Fig. 2I & Supplementary Fig. 8F in the manuscript). Moreover, plasma GDF-15 levels strongly correlated with *Gdf15* expression levels in liver, but not in adipose tissue unlike in the mouse models of obesity (Fig. 1C & 2M in the manuscript). In the experiment alluded to by the reviewer, we also observed a correlation between plasma GDF-15 levels and *Gdf15* expression in liver (Fig. R1). The coefficient of determination (R^2) calculated by simple linear regression was 0.8291 (Fig. R1), meaning that *Gdf15* expression in liver might explain >80% of variations in plasma GDF-15 concentrations which strongly support that the liver is the major source of GDF-15 in the CDAA-induced MASH model.

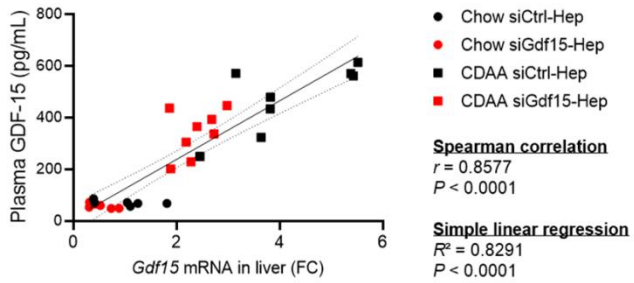

Figure R1. Association of plasma GDF-15 levels with Gdf15 expression in liver.
Mice were fed a chow or CDAA diet for 4 weeks and tail vein injected with invivofectamine-siRNA complex (1.5 mg/kg siRNA) 4 days prior sacrifice (n = 6-8/group). Correlation between plasma GDF-15 concentrations and *Gdf15* mRNA expression in liver. FC, fold change.

Hepatocytes are the key source of hepatic GDF-15: In figure 6D in the manuscript, we showed that the hepatocyte fraction expresses approximately 3-fold more *Gdf15* than non-hepatocytes (NPCs, non-parenchymal cells) in naive mouse livers. These results are in agreement with previous works showing that GDF-15 is mostly expressed by hepatocytes[3,4]. Moreover, *Gdf15* expression increased in hepatocytes from mice fed the MASH-inducing diet (Fig. 6E in the manuscript) to the same extent than in whole liver (Fig. 2I & 2K in the manuscript). For these reasons and given that hepatocytes represent the large majority of the liver cells, we assume that hepatocytes are likely the main source of GDF-15 in liver.

In the experiment pointed out by the reviewer (Fig. 6F in the manuscript), we silenced *Gdf15* in hepatocytes by using invivofectamine 3.0 and siRNA targeting Gdf15. We obtained a knockdown of 43% in livers from CDAA-fed mice (Fig. 6F in the manuscript) and a respective decrease of 29% in plasma GDF-15 concentrations (Fig. 6G in the manuscript), showing a proportional response between mRNA silencing and circulating level of GDF-15. Indeed, we observed a nearly linear relationship between *Gdf15* silencing in liver and plasma GDF-15 concentrations (Fig. R1). Yet, and as rightly indicated by the reviewer, we did not fully suppress the CDAA-induced *Gdf15* expression in liver or plasma GDF-15 concentrations. This incomplete suppression arises, at least partially, from intrinsic technical reasons. Indeed, siRNA-based technology does not achieve 100% silencing even in optimal condition. Moreover, the transfection reagent likely does not deliver the optimal amount of siRNAs in every single hepatocyte, also contributing to the incomplete silencing in hepatocytes. Importantly, as the original purpose of the experiment was to demonstrate the functional contribution of the hepatocytes to GDF-15 production in MASH, an incomplete knockdown was not an issue as far as we observed an unambiguous impact on plasma GDF-15 levels, which is shown in figure 6G in the manuscript (adjusted *P* value 0.0084).

Although not very likely, this however does not fully rule out a contribution of a hepatic cell type other than hepatocyte to GDF-15 production as mentioned by the reviewer. We have now incorporated these considerations in the manuscript (lines 368-370).

Comment 2: I like the response in my comment 2.

Response 2: We are delighted that reviewer appreciated our response.

Comment 3: Inactivation of Gdf15 in macrophages in BMT model did not reduce plasma GDF-15 concentrations in later phase in fig. 5J. What are the possible reasons?

Response 3: These results were briefly discussed in lines 489-491 & 516-518 and have now been further detailed for more clarity (lines 491-494 & 518-519).

At an early stage of obesity, and before liver complications develop, macrophage infiltration into adipose tissue leads to increased plasma GDF-15 concentrations (Fig. 2 to 5 in the manuscript). In the BMT experiment, this is observed after 4 weeks of HFD feeding when mice displayed a modest, but significant, increase of plasma GDF-15 concentrations (Fig. 5G & 5J in the manuscript). *Gdf15*$^{-/-}$ BMT mice, which gain similar weight as *Gdf15*$^{+/+}$ BMT mice at 4 weeks, exhibited lower levels of plasma GDF-15 because of their deficiency of *Gdf15* in macrophages. As in mice, obesity alone modestly increased plasma GDF-15 concentrations in patients (Fig. 2E in the manuscript).

At advanced stages of obesity, when liver complications develop, the liver is becoming an increasingly important source of GDF-15. This is demonstrated in the extreme obesity-independent MASH-like model (CDAA diet) where a robust increase of plasma GDF-15 concentrations through liver production is observed (Fig. 2J in the manuscript). Similarly, MASH robustly increased plasma GDF-15 concentrations in patients matched for obesity and T2D (Fig. 2E in the manuscript), demonstrating that the impact of liver pathology, may even exceed the effect of obesity on plasma GDF-15 elevation.

While macrophage infiltration regulates *Gdf15* expression in adipose tissue, it does not in liver (Fig. 6 in the manuscript). Accordingly, the *Gdf15* deficiency in immune cells in *Gdf15*$^{-/-}$ BMT mice had not impact on HFD-induced hepatic *Gdf15* expression at later stages (Fig. 5K in the manuscript). Since HFD induces *Gdf15* expression in liver at later stages and that *Gdf15* deficiency in immune cells has no effect on it, it is very likely that liver-derived GDF-15 might progressively attenuates the difference in plasma GDF-15 levels observed between *Gdf15*$^{+/+}$ and *Gdf15*$^{-/-}$ BMT mice at early stages (Fig. 5J in the manuscript), explaining why the difference between genotype did not persist over time. The role of liver-derived GDF-15 to explain this loss of difference is even more likely in our experimental setting than *Gdf15*$^{-/-}$ BMT mice developed more severe obesity, liver complications and expressed higher *Gdf15* levels in liver compared to *Gdf15*$^{+/+}$ BMT mice (Fig. 5G-H, Supplementary Fig. 7G-I, Fig. 5K in the manuscript). Although not statistically significant, HFD feeding increased liver *Gdf15* expression 2.5-fold in *Gdf15*$^{+/+}$ and 3.0-fold in *Gdf15*$^{-/-}$ BMT mice (Fig. 5K in the manuscript). In agreement with this observation, we also observed that the two transcription factors that we identified as regulating *Gdf15* in liver (*Tfeb* and *Ddit3*) were higher in *Gdf15*$^{-/-}$ BMT mice (Fig. R2). We believe therefore that the difference in plasma GDF-15 levels between *Gdf15*$^{+/+}$ and *Gdf15*$^{-/-}$ BMT mice at early stages of obesity is attenuated over time by liver production following the development of liver complications.

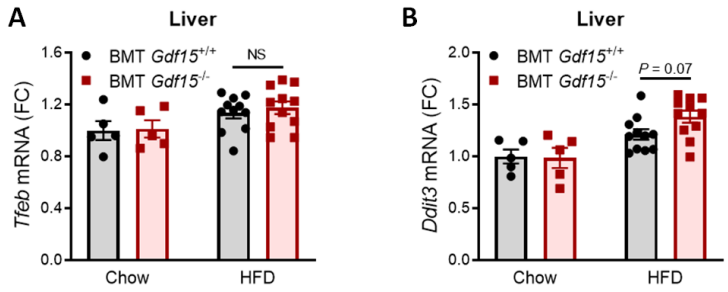

Figure R2. Expression of *Tfeb* and *Ddit3* in liver.
After bone marrow transplantation (BMT) from Gdf15$^{+/+}$ or Gdf15$^{-/-}$ mice, reconstituted mice were fed a chow or a HFD for 12 weeks (n = 16/genotype; 5 chow and 11 HFD). **(A)** *Tfeb* mRNA expression levels in liver. **(B)** *Ddit3* mRNA expression levels in liver.

Comment 4: What about food intake data for the models injected with siRNA targeting Tfeb and Ddit3?

Response 4: We thank the reviewer once again for suggesting this experiment in the previous revision (Comment 5: "*How about GDF15 levels in MASH when deletion of both TFEB and DDIT3 in mice?"*). This substantially improved our manuscript by confirming the *in vivo* relevance of our findings. Unfortunately, food intake was not measured in this experiment due to both the design of the experiment and technical reasons detailed below.

The experiment was designed to demonstrate the direct role of TFEB and DDIT3 on *Gdf15* expression in liver and plasma GDF-15 concentrations. We therefore opted for an acute deletion by siRNA for 4 days in order to bypass the chronic effects of a long-term deletion since TFEB and DDIT3 are known to play roles in liver lipid metabolism and MASLD (ref). Since a minimum of 24 to 48 hours is usually required to reach efficient silencing by siRNA, the time window for measuring food intake was extremely short to obtain accurate data.

Indeed, food intake is more reliable when measured on few consecutive days (cumulative food intake). Moreover, unlike chow and HFD feeding, which generate minor food spillage, CDAA diet feeding produces massive spillage rendering accurate measurements of food intake difficult. Finally, water contained added sugar (further details provided in methods in the manuscript), providing an additional source of energy to the CDAA diet, which adds further variability of measurement (water bottle leakage, …).

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REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

I think the authors answered all my questions and concerns properly.