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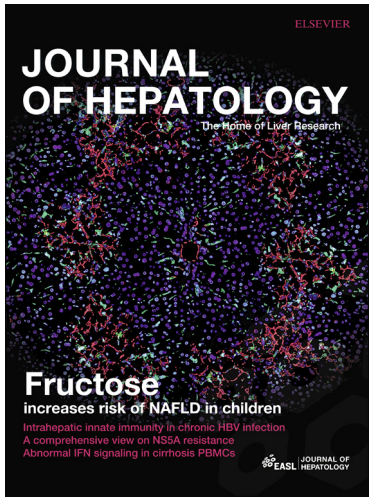

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Change in hepatic fat content measured by MRI does not predict treatment-induced histological improvement of steatohepatitis

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Short Title: Longitudinal liver fat

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Author Contribution

F.B. contributed to patient recruitment and follow-up, data acquisition and interpretation, statistical analysis, and writing and editing of the manuscript. D.B., and R.L., contributed to patient recruitment and follow-up, data acquisition, and critical revision of the manuscript. J.L. contributed to the reading of liver biopsies and critical revision of the manuscript. K.C. contributed to the study design and funding, patient recruitment and follow-up, data acquisition and interpretation, and critical revision and editing of the manuscript. All authors reviewed and

approved the final manuscript. F.B. and K.C. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

LAY SUMMARY

Quantification of liver fat by MRI is currently used to assess response to treatment in patients with fatty liver (NAFLD), with the assumption that improvement in liver fat translate into less inflammation, necrosis, and fibrosis in the liver. However, in this article we showed that changes in liver fat do not necessarily translate into changes in these parameters. This means that MRI may not be as useful as previously believed to assess treatment response in patients with fatty liver.

ABSTRACT

Background & Aims

Proof-of-concept studies frequently assess changes in intrahepatic triglyceride (IHTG) content by MR-based techniques as a surrogate marker of histology. The aim of this study was to establish how reliable this strategy is to predict changes in liver histology in patients with NASH.

Methods

Patients with NASH who had participated in our prior randomized controlled trials of pioglitazone with complete paired data for IHTG content by magnetic resonance spectroscopy and liver histology were included in the study.

Results

A total of 121 patients were included. Changes in IHTG were assessed in several ways: as a continuous variable (correlations), as categorical groups (IHTG change ≥0%; or IHTG reduction of 1-30%; 31-50%; 51-70%; or >70%), and in a

binomial way as steatosis resolution or not (defined as achieving IHTG<5.56%). Changes in IHTG correlated with steatosis on histology (r = 0.54; p<0.01). However, the magnitude of IHTG reduction was not associated with the rate of response of the primary histological outcome (2-point improvement in the NAFLD activity score from two different parameters, without worsening of fibrosis) or resolution of NASH without worsening of fibrosis, neither in patients receiving pioglitazone nor placebo. Changes in lobular inflammation, hepatocyte ballooning, or liver fibrosis were also independent of changes in IHTG, irrespective of treatment arm. Steatosis resolution was not associated with better histological outcomes either.

Conclusions

Changes in IHTG predicts changes in steatosis but not of other liver histological parameters. This implies that IHTG response to treatment should be interpreted with caution as changes may not be as reliable as previously believed to determine overall clinical efficacy of novel treatments in patients with NASH.

In recent years, there has been a growing interest in the discovery of new pharmacological agents for the treatment of nonalcoholic fatty liver disease (NAFLD). This recent interest responds to an alarming rise in its prevalence and, more importantly, to its associated risk of progression to end-stage liver disease (1). Several randomized, controlled trials have recently been completed (2-8), and many more are still ongoing (9).

A percutaneous liver biopsy remains the gold-standard for the diagnosis of NASH and is required by the FDA for a new drug indication (10, 11). However, early proof-of-concept studies often use changes in intrahepatic triglyceride (IHTG) content by either magnetic resonance imaging-proton density fat fraction (MRI-PDFF) or proton magnetic resonance spectroscopy (^{1}H-MRS) as a surrogate marker of resolution of NASH and/or improvement in fibrosis (12-14).

Intrahepatic triglyceride content measured by either ^{1}H-MRS or MRI-PDFF correlates well with steatosis assessed by histology (15-17). Studies by Noureddin et al (16) and Middleton et al (17) demonstrated that longitudinal changes in IHTG content measured by magnetic resonance techniques strongly correlated with changes in steatosis on histology. However, while it is generally assumed that changes in steatosis may likely be followed by similar changes in other histological parameters, as well as resolution of NASH, there is a paucity of information regarding this relationship. Moreover, the degree of IHTG content reduction required to improve lobular inflammation, hepatocyte ballooning,

fibrosis, or to achieve resolution of NASH, remains unclear. In a small (n=35) short-term (24 weeks) study, Patel et al showed that histologic responders had a significantly higher reduction of liver fat compared to non-responders (18). However, the authors defined histological response as a reduction in 2 points of the NAS score, which includes steatosis grade as a parameter. Therefore, it is possible that a reduction in the steatosis grade could have significantly confounded the association. More recently, the same group reported that changes in IHTG content during the phase II study of selonsertib did not predict changes in hepatocyte ballooning or fibrosis (19).

The aim of this study was to assess in a large cohort of patients followed for 6 to 18 months the relationship between the degree of intrahepatic triglyceride content reduction and improvement in other histological parameters and resolution of NASH.

RESEARCH DESIGN AND METHODS

Subjects

A total of 121 subjects were recruited from Gainesville, FL and San Antonio, TX that participated in one of our three randomized, controlled, trials assessing pioglitazone in patients with NASH (4, 20, 21). Patients not included in this report were either randomized to vitamin E (14%), did not complete all follow-up visits or procedures (16%), refused the final liver biopsy (1%), or did not have paired ^{1}H-MRS measurements available (24%).

Detailed inclusion and exclusion criteria are available in prior reports (4, 20, 21). Briefly, patients with a diagnosis of biopsy-proven NASH, with or without type 2 diabetes mellitus (T2DM) were included in the randomized, controlled trials. Main exclusion criteria were any other causes of liver disease, such as viral hepatitis, autoimmune hepatitis, Wilson's disease, hemochromatosis, drug-induced hepatitis, a history of significant alcohol use (≥30 grams/day in males and ≥20 grams/day in females), as detailed elsewhere (4, 20, 21). Informed written consent was obtained from each patient prior to participation, and the study was approved by the IRBs from both universities (University of Florida, Gainesville, FL and University of Texas Health Science Center at San Antonio, San Antonio, TX).

Study design

This is a longitudinal study of patients with complete ^{1}H-MRS and histological data from 3 previously published randomized controlled trials (4, 20, 21). As part of those studies, at baseline and after treatment patients underwent the following assessments: (a) liver ^{1}H-MRS to quantify hepatic triglyceride content, (b) oral glucose tolerance test to measure insulin resistance and establish the diagnosis of T2DM according to the American Diabetes Association criteria (22), (c) dual energy x-ray absorptiometry (DEXA) to measure total body fat (TBF), and (d) a percutaneous liver biopsy to establish (or rule out) the diagnosis of NASH. Patients were originally randomized to either placebo or active treatment: pioglitazone monotherapy in two studies (4, 20), or combined with vitamin E in

another (21). In this report, 62 patients participated in an 18-month trial comparing pioglitazone vs. placebo (4), 22 patients participated in an 18-month trial assessing the use of vitamin E alone and combination therapy with pioglitazone and vitamin E (21), and 37 patients participated in a 6-month trial comparing pioglitazone vs. placebo (20). Study design was similar for all studies, except for one study having a shorter duration of therapy (20). In this study we report only on those patients that received either placebo or pioglitazone during their participation in the study (patients randomized to vitamin E alone were excluded).

Liver fat content by ^{1}H-MRS

Proton magnetic resonance spectroscopy of the liver was acquired using methodology previously validated (15). Briefly, three 30 mm x 30 mm x 30 mm liver locations were selected avoiding vessels and bile ducts. Liver fat content was calculated as fat fraction (area under the curve [AUC] fat peak/[AUC fat peak + water peak]) using commercial software (NUTS, Acorn NMR). Measurements were corrected for T1 and T2 relaxation as previously described (23). While the 6-month trial (20) used a 1.9T scanner (Elscint Prestige), the 18-month trials used 3T machines (4, 21).

Liver biopsy

An ultrasound-guided liver biopsy was performed in all patients at baseline and at the end of the study. Biopsies were centrally read by 2 pathologists who were

unaware of the subjects' identity, time point (i.e., before or after therapy) or any clinical information. Histologic characteristics for the diagnosis of NASH were determined using standard criteria (24, 25). Histological response after therapy was defined as resolution of NASH (ballooning of 0 and inflammation of 0-1) without worsening of fibrosis. Data was also analyzed considering a reduction in 2 points in the NAFLD activity score (NAS) from 2 different parameters without worsening of fibrosis as a secondary histological outcome. The mean length of liver biopsies was 16.5 ± 0.7mm, 13% were <10mm, 30% were between 10-14.9mm, 30% were between 15-19.9mm, and 27% were ≥20mm.

Statistical analysis

Values for continuous variables are reported as mean ± standard deviation of the mean and as number (percentage) for categorical variables. Comparison among groups was performed using ANOVA (Bonferroni's pos hoc analysis for pairwise comparisons) or Kruskal-Wallis for continuous variables according to their distribution, or Pearson's Chi-square or Fisher's Exact Test for categorical variables. A p-value of <0.05 (two-tailed) was considered statistically significant. All statistical calculations were performed using Stata 11.1 (StataCorp LP, TX, USA) and JMP version 11 (SAS Institute Inc., Cary, NC).

RESULTS

Subject Characteristics

Demographic and clinical information of patients is described in **Table 1**, where they were divided based on the relative change of IHTG content after therapy (no change or increase; reduction of 1-30%; reduction of 31-50%; reduction of 51-70%; and reduction of >70%). There were no statistically significant differences in baseline clinical variables among groups with different IHTG content reduction, except in plasma ALT levels. In **Supplemental Table 1**, we have also summarized baseline clinical information of patients, but dividing them based on histological response (responders vs. non-responders) depending on the trial they had participated in. The only significant differences observed in responders vs. non-responders were worse baseline histology (i.e., inflammation, ballooning, and fibrosis) and higher prevalence of T2DM among responders of the longer clinical trial, as previously reported (26). Similarly, fasting plasma glucose levels were higher among responders of the 6-month clinical trial. Of note, the degree of steatosis at baseline, based on either ^{1}H-MRS or histology, were similar between histological responders and non-responders. While age was statistically different among groups in the 18-month clinical trials, this difference had only minor clinical significance.

Relationship between changes in IHTG content and histological changes

In **Figure 1**, we analyzed the rate of resolution of NASH without worsening of fibrosis based on the relative reduction of IHTG measured by ^{1}H-MRS. As can be observed, no significant changes in histological response were observed with increasing response in steatosis reduction by ^{1}H-MRS in either patients

randomized to placebo (**Figure 1A**) or those taking pioglitazone (**Figure 1B**). In addition, we have also presented the data using a reduction of 2 points in the NAS (from at least 2 different parameters) and no worsening of fibrosis, as another histological outcome (**Figure 1C** and **1D**). Again, no clear association was observed between IHTG content reduction and histological response. No significant differences were observed in resolution of NASH when patients with a reduction of more than 30% of IHTG content were compared to those with less reduction (among patients randomized to pioglitazone: 49 vs. 33%, p=0.67; among placebo users: 12 vs. 6%, p=0.64). Similarly, reduction of 2 points in the NAS (from at least 2 different parameters) and no worsening of fibrosis was also similar between patients achieving vs. not achieving 30% reduction of IHTG (pioglitazone: 58 vs. 50%, p=0.99; placebo: 20 vs. 15%, p=0.73). To assess whether IHTG above normal (i.e., >5.56%) may have a permissive effect on inflammation and hepatocyte ballooning, we also assessed the rate of histological response among patients achieving vs. not achieving steatosis resolution (i.e., achieving an IHTG content below 5.56%). As detailed in **Figure 2,** no significant differences were observed in resolution of NASH or reduction of NAS score (without worsening of fibrosis) based on the presence of steatosis resolution. To further analyze the role of IHTG content reduction in the prediction of resolution of NASH without worsening of fibrosis, we calculated the performance of each specific cut-off point of IHTG reduction (**Table 2**). No specific cut-off point of IHTG content reduction was able to accurately predict the presence of resolution of NASH without worsening of fibrosis. For example, a

reduction of at least 30% of IHTG content, an outcome usually used in clinical trials, was only associated with a specificity of 41% and PPV of 38%. In **Supplemental Table 2** we repeated this analysis including only patients that received active treatment with pioglitazone. Again, no specific cut-off point of IHTG content reduction was able to predict resolution of NASH.

In **Figure 3**, rate of improvement in each of the histological parameters (lobular inflammation, hepatocyte ballooning, and fibrosis) was plotted for groups with different amounts of relative reduction of IHTG content. As can be observed, no significant associations were detected between reduction of IHTG and these histological parameters. Finally, we also assessed whether steatosis resolution (reaching an IHTG below 5.56%) could be associated with better histological response. Patients with steatosis resolution had similar rates of resolution of NASH irrespectively of treatment arm (51% vs. 41% for active treatment with pioglitazone and 13% vs. 7% for the placebo arm, both p=0.60).

In **Figure 4**, we have summarized inflammation (panels A-B), hepatocyte ballooning (panels C-D) and fibrosis (panels E-F) changes based on the presence or absence of steatosis resolution. Only lobular inflammation in patients taking pioglitazone appeared to have a modest difference among patients reaching steatosis resolution versus not doing so (p=0.048). All other histological changes in patients randomized to either placebo or pioglitazone were similar between patients reaching and not reaching steatosis resolution.

Relationship between changes in IHTG content and surrogate markers of liver disease

Relative changes observed in IHTG content after follow-up correlated with changes in plasma ALT (r=0.23, p=0.008), but not with changes in AST (r=0.13, p=0.14), or CK-18 (r=0.07, p=0.50). Of note, plasma ALT was more tightly related to changes in ballooning (r=0.41, p<0.001) than changes in IHTG content measured by ^{1}H-MRS. Among patients with steatosis resolution, improvements of plasma ALT and AST were of similar magnitude to those observed in patients not reaching steatosis resolution as defined above (**Figure 5**).

Among patients randomized to placebo, changes in IHTG content after follow-up were significantly associated with weight loss (r=0.33, p=0.01). Moreover, weight loss was also significantly associated with changes in hepatocyte ballooning (r=0.42, p=0.008), and fibrosis (r=0.36, p=0.02). However, changes in IHTG content were not associated with changes in other metabolic parameters, such as hemoglobin A1c (r=-0.04, p=0.83), fasting plasma insulin (r=-0.26, p=0.14), or plasma triglycerides (r=0.23, p=0.17) concentration. Reduction of IHTG content was associated with a significant increase in plasma HDL-C levels (r=-0.33, p=0.04). In the cohort of patients receiving pioglitazone, we observed no significant associations between changes in IHTG content and changes in hemoglobin A1c (r=-0.15, p=0.34), fasting plasma insulin (r=-0.02, p=0.89),

plasma triglycerides (r=-0.09, p=0.55), or plasma HDL-C (r=0.04, p=0.81) concentration.

Sensitivity Analysis

Due to differences in the 6-month trial compared to the 18-month trial, we have performed a sensitivity analysis repeating the above calculations, but excluding the subgroup of patients that participated in the 6-month study. As can be observed in **Figure 6A-B**, no differences were observed in the overall results. Due to the relatively small sample size (n=37), the same kind of analysis could not be performed for the 6-month trial alone. In addition, as prior studies have analyzed patients on active medication and on placebo combined (18, 19), we have also presented this analysis combining the 121 patients (**Figure 6C-D**). As evidenced in this figure, when combining patients on placebo with those on active medication, it appears that reduction on IHTG content is associated with resolution of NASH without worsening of fibrosis.

DISCUSSION

Proton magnetic resonance spectroscopy (^{1}H-MRS) and magnetic resonance imaging-proton density fat fraction (MRI-PDFF) have consolidated as non-invasive gold-standards for the measurement of IHTG content in patients with NAFLD (15, 16). Due to their high reliability and reproducibility, MRI-based techniques have also consolidated as primary outcome measures in early proof-

of-concept NASH clinical trials (i.e., phase I or II), working as surrogate markers of histological response (i.e., for hepatocyte necrosis and inflammation) (12, 13, 18). However, in the current study, we suggest that changes in IHTG measured after 6 or 18 months in patients receiving either placebo or pioglitazone correlate less strongly with histological changes than previously believed. If confirmed in larger studies and with other therapeutic agents, these results indicate that quantification of IHTG content should be used with caution to assess overall response in patients with NASH.

In a prior cross-sectional study, we have reported that IHTG accumulation beyond 4-8% was associated with maximal metabolic impairment (23). Moreover, we showed in that study that further IHTG accumulation was not associated with worse liver histology. In the current work, we go further to suggest that the magnitude of IHTG change with treatment is not strongly correlated with histological or metabolic changes, at least in patients receiving placebo or thiazolidinedione therapy. This is in apparent discordance with two prior studies that have assessed the longitudinal relationship between IHTG and liver histology. In a 24-week study in 35 patients with biopsy-proven NASH by Patel et al (18), the investigators noted that changes in IHTG were associated with significant reductions in the NAFLD activity score. However, the NAFLD activity score (NAS) includes steatosis as one of its parameters. Therefore, comparing changes of IHTG with changes in NAS are likely to be correlated based on similar changes in steatosis (i.e., collinearity). Of note, these results were

comparable to ours in that there was no association between a reduction in IHTG and individual histological parameters such as inflammation or ballooning (18). We found only a borderline positive relationship between changes in IHTG and lobular inflammation, similar to that observed in patients treated with selonsertib and/or simtuzumab (considered a placebo), and where changes in MRI-PDFF were not strong predictors of improvement in hepatocyte ballooning or fibrosis (19). In that study, the AUC to predict NAS response was identical to that to predict steatosis response (both were 0.70), strongly suggesting collinearity between both measurements. In order to avoid this in the current work, we used resolution of NASH without worsening of fibrosis as the histological outcome, which is less dependent of changes in steatosis.

Another potential reason for the positive findings in prior studies was the combination of patients on placebo (or simtuzumab) with patients on active treatment (18, 19). In our cohort of patients, when analyses were performed combining patients receiving placebo and active treatment, results were misleading as they suggested that there was a strong association between changes in IHTG content and histological changes. This is expected, as most patients on pioglitazone had reductions on IHTG content (45 out of 63 had >50% reduction), on insulin resistance, A1c, and histological improvement. On the contrary, patients on placebo showed no changes in IHTG, insulin resistance, A1c, histology. Therefore, when combining patients on pioglitazone and placebo, associations between metabolic variables result significant as a result of having 2

heterogeneous and extreme populations. This results in a significant overestimation of the association coefficient. In fact, no association was observed between changes in IHTG content and histological response when a two-way ANOVA was performed adjusting for treatment arm. Moreover, stratifying patients based on whether they received placebo or pioglitazone resulted in the absence of any significant association between IHTG content changes and histological improvement (**Figures 1-3**). Finally, the relationship between changes in steatosis and changes in other histological features may vary depending on the mechanism of action of each intervention. Lifestyle intervention appears to impact similarly individual histological parameters (steatosis, ballooning and inflammation) (27, 28) and a recent study reported that the MRI-PDFF response was associated with resolution of NASH, but it did not provide a quantitative analysis of the role of IHTG content reduction (29).

The most straightforward implication of these findings is that the use of MRI-PDFF or ^{1}H-MRS cannot be considered as useful as currently believed to assess histological response to treatment. In addition, it calls for a paradigm shift about individual histological parameters improving in tandem (i.e., changes in steatosis expected to be followed by changes in necroinflammation). For instance, rosiglitazone (an exclusive PPARγ activator) reduced steatosis but failed to have a significant effect on inflammation or hepatocyte ballooning (30, 31). In early drug development there is some expectation that fibrosis is also more likely to improve, at least to some extent, with greater reductions in liver fat. But the

discordance regarding changes in steatosis and fibrosis is now clear. Cenicriviroc treatment was associated with some improvement in fibrosis, but no changes in steatosis, inflammation or ballooning (7). In the GOLDEN-505 study assessing elafibranor at 2 different doses against placebo, changes in fibrosis were not correlated with changes in steatosis (3). More recently, obeticholic acid improved fibrosis without any correlation with steatosis and no significant resolution of NASH (32).

The reason(s) why improvements in IHTG do not more readily translate to other histological improvement remains unclear. Such a dissociation was also evident in our prior work where disease severity was not different throughout the range of IHTG ranging from 6% to ≥30%, suggesting that steatosis *per se* is more of a "trigger" than the determinant of disease severity in NASH (23). Of note, complete steatosis resolution (≤5.56%) was not a strong indicator of resolution of NASH (**Figure 2**).

It is possible that IHTG-driven lipotoxicity induces changes in hepatocytes, stellate and Kupffer cells, which are not reversible by triglyceride reduction alone, and that they may require additional hormonal or downstream effects on pathways linked to mitochondrial fatty acid oxidation, insulin/lipid signaling, or regulation of inflammatory pathways. In support of this concept, while rosiglitazone and pioglitazone reduce steatosis, only pioglitazone induces improvements in inflammation and ballooning, suggesting accessory

mechanisms (4, 20, 30, 31). Another potential hypothesis is that IHTG reduction may require a certain amount of time to promote histological changes. However, in our placebo-controlled studies, paired biopsies performed at 6 or 18 months of treatment (4, 20) led to similar histologic and imaging changes, and histological improvement was the same even after 36 months (4), suggesting that time *per se* is not a reasonable explanation for the differences observed between IHTG content changes and histology. Moreover, our sensitivity analysis did not find any differences in results when analyses were repeated separating patients based on duration of follow-up (6 vs. 18 months). However, it is still possible that histological changes may require even a longer period of time to settle after IHTG reduction.

One may speculate based on a growing body of literature, that triglycerides *per se* are not harmful and that they may not even be correlated with the accumulation of toxic lipid metabolites that drive hepatocyte lipotoxicity (33, 34). Indeed, they may even be protective for the liver, acting as a buffer against the accumulation of lipotoxic diacylglycerols (DAGs) (35), ceramides (36) or other lipid species (37). Both DAGs and ceramides are emerging as the link between overnutrition and excess FFA/energy supply to the liver and hepatocyte lipotoxicity in NASH (38). Different groups have reported that hepatocyte DAGs are increased in obese patients with NASH (39, 40). Also, an increased concentration of ceramide both in plasma and within hepatocytes has been associated with impaired mitochondrial fatty acid oxidation and inflammation in

patients with obesity and T2DM (41, 42). Taking this building evidence into account, it is easier to reconcile why a reduction of IHTG may not directly translate into improvements in inflammation, ballooning, or fibrosis. The impact of weight loss or pharmacological agents on these lipotoxic metabolites has not been measured in liver tissue in humans, but in animal models of NASH, both GLP-1RAs (43) and pioglitazone (44) lead to a reduction of DAGs, ceramides and acylcarnitines and metabolic improvement.

In the current work, we have shown that changes in IHTG content after treatment with pioglitazone or placebo do not predict histological or metabolic changes. The clinical implication of our findings is that MRI-based imaging may not be as reliable as previously believed to guide about the clinical efficacy of a given intervention in patients with NASH. However, larger studies and examination of agents with different mechanisms of action are needed to confirm whether this observation is generalizable. Until such studies become available, randomized controlled trials using IHTG as a surrogate endpoint for broader histological response should be interpreted with caution.

REFERENCES

1. Bril F, Cusi K. Management of nonalcoholic fatty liver disease in patients with type 2 diabetes: A call to action. Diabetes Care 2017;40:419-430.
2. Neuschwander-Tetri BA, Loomba R, Sanyal AJ, Lavine JE, Van Natta ML, Abdelmalek MF, Chalasani N, et al. Farnesoid X nuclear receptor ligand

obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. Lancet 2015;385:956-965.

3. Ratziu V, Harrison SA, Francque S, Bedossa P, Lehert P, Serfaty L, Romero-Gomez M, et al. Elafibranor, an agonist of the peroxisome proliferator-activated receptor-alpha and -delta, induces resolution of nonalcoholic steatohepatitis without fibrosis worsening. Gastroenterology 2016;150:1147-1159 e1145.
4. Cusi K, Orsak B, Bril F, Lomonaco R, Hecht J, Ortiz-Lopez C, Tio F, et al. Long-term pioglitazone treatment for patients with nonalcoholic steatohepatitis and prediabetes or type 2 diabetes mellitus: A randomized trial. Ann Intern Med 2016;165:305-315.
5. Sanyal AJ, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, Neuschwander-Tetri BA, et al. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. N Engl J Med 2010;362:1675-1685.
6. Armstrong MJ, Gaunt P, Aithal GP, Barton D, Hull D, Parker R, Hazlehurst JM, et al. Liraglutide safety and efficacy in patients with non-alcoholic steatohepatitis (LEAN): a multicentre, double-blind, randomised, placebo-controlled phase 2 study. Lancet 2016;387:679-690.
7. Friedman SL, Ratziu V, Harrison SA, Abdelmalek MF, Aithal GP, Caballeria J, Francque S, et al. A randomized, placebo-controlled trial of cenicriviroc for treatment of nonalcoholic steatohepatitis with fibrosis. Hepatology 2018;67:1754-1767.

8. Harrison SA, Abdelmalek MF, Caldwell S, Shiffman ML, Diehl AM, Ghalib R, Lawitz EJ, et al. Simtuzumab is ineffective for patients with bridging fibrosis or compensated cirrhosis caused by nonalcoholic steatohepatitis. Gastroenterology 2018;155:1140-1153.
9. Younossi ZM, Loomba R, Rinella ME, Bugianesi E, Marchesini G, Neuschwander-Tetri BA, Serfaty L, et al. Current and future therapeutic regimens for nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. Hepatology 2018;68:361-371.
10. Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, Harrison SA, et al. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. Hepatology 2018;67:328-357.
11. Sanyal AJ, Friedman SL, McCullough AJ, Dimick-Santos L, American Association for the Study of Liver D, United States F, Drug A. Challenges and opportunities in drug and biomarker development for nonalcoholic steatohepatitis: findings and recommendations from an American Association for the Study of Liver Diseases-U.S. Food and Drug Administration Joint Workshop. Hepatology 2015;61:1392-1405.
12. Loomba R, Sirlin CB, Ang B, Bettencourt R, Jain R, Salotti J, Soaft L, et al. Ezetimibe for the treatment of nonalcoholic steatohepatitis: assessment by novel magnetic resonance imaging and magnetic resonance elastography in a randomized trial (MOZART trial). Hepatology 2015;61:1239-1250.

13. Petit JM, Cercueil JP, Loffroy R, Denimal D, Bouillet B, Fourmont C, Chevallier O, et al. Effect of liraglutide therapy on liver fat content in patients with inadequately controlled type 2 diabetes: The lira-NAFLD study. J Clin Endocrinol Metab 2017;102:407-415.
14. Cui J, Philo L, Nguyen P, Hofflich H, Hernandez C, Bettencourt R, Richards L, et al. Sitagliptin vs. placebo for non-alcoholic fatty liver disease: A randomized controlled trial. J Hepatol 2016;65:369-376.
15. Bril F, Ortiz-Lopez C, Lomonaco R, Orsak B, Freckleton M, Chintapalli K, Hardies J, et al. Clinical value of liver ultrasound for the diagnosis of nonalcoholic fatty liver disease in overweight and obese patients. Liver Int 2015;35:2139-2146.
16. Noureddin M, Lam J, Peterson MR, Middleton M, Hamilton G, Le TA, Bettencourt R, et al. Utility of magnetic resonance imaging versus histology for quantifying changes in liver fat in nonalcoholic fatty liver disease trials. Hepatology 2013;58:1930-1940.
17. Middleton MS, Heba ER, Hooker CA, Bashir MR, Fowler KJ, Sandrasegaran K, Brunt EM, et al. Agreement between magnetic resonance imaging proton density fat fraction measurements and pathologist-assigned steatosis grades of liver biopsies from adults with nonalcoholic steatohepatitis. Gastroenterology 2017;153:753-761.
18. Patel J, Bettencourt R, Cui J, Salotti J, Hooker J, Bhatt A, Hernandez C, et al. Association of noninvasive quantitative decline in liver fat content on MRI

with histologic response in nonalcoholic steatohepatitis. Therap Adv Gastroenterol 2016;9:692-701.

19. Jayakumar S, Middleton MS, Lawitz EJ, Mantry PS, Caldwell SH, Arnold H, Mae Diehl A, et al. Longitudinal correlations between MRE, MRI-PDFF, and liver histology in patients with non-alcoholic steatohepatitis: Analysis of data from a phase II trial of selonsertib. J Hepatol 2019;70:133-141.
20. Belfort R, Harrison SA, Brown K, Darland C, Finch J, Hardies J, Balas B, et al. A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis. N Engl J Med 2006;355:2297-2307.
21. Bril F, Biernacki DM, Lomonaco R, Kalavalapalli S, Subbarayan SK, Lai J, Tio F, et al. Role of vitamin E for nonalcoholic steatohepatitis in patients with type 2 diabetes: A randomized controlled trial. Diabetes Care 2019;42:1481-1488.
22. Summary of revisions: Standards of medical care in diabetes-2019. Diabetes Care 2019;42:S4-S6.
23. Bril F, Barb D, Portillo-Sanchez P, Biernacki D, Lomonaco R, Suman A, Weber MH, et al. Metabolic and histological implications of intrahepatic triglyceride content in nonalcoholic fatty liver disease. Hepatology 2017;65:1132-1144.
24. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005;41:1313-1321.

25. Yeh MM, Brunt EM. Pathological features of fatty liver disease. Gastroenterology 2014;147:754-764.
26. Bril F, Kalavalapalli S, Clark VC, Lomonaco R, Soldevila-Pico C, Liu IC, Orsak B, et al. Response to pioglitazone in patients with nonalcoholic steatohepatitis with vs without type 2 diabetes. Clin Gastroenterol Hepatol 2018;16:558-566 e552.
27. Promrat K, Kleiner DE, Niemeier HM, Jackvony E, Kearns M, Wands JR, Fava JL, et al. Randomized controlled trial testing the effects of weight loss on nonalcoholic steatohepatitis. Hepatology 2010;51:121-129.
28. Vilar-Gomez E, Martinez-Perez Y, Calzadilla-Bertot L, Torres-Gonzalez A, Gra-Oramas B, Gonzalez-Fabian L, Friedman SL, et al. Weight loss through lifestyle modification significantly reduces features of nonalcoholic steatohepatitis. Gastroenterology 2015;149:367-378 e365; quiz e314-365.
29. Harrison S, Guy C, Taub R, Bashir M. MRI-PDFF response in MGL-3196 and placebo treated patients predicts reduction in ballooning and inflammation components of NAS and NASH resolution in a 36-week serial liver biopsy study. J Hepatol 2019;70:e791-e792.
30. Ratziu V, Giral P, Jacqueminet S, Charlotte F, Hartemann-Heurtier A, Serfaty L, Podevin P, et al. Rosiglitazone for nonalcoholic steatohepatitis: one-year results of the randomized placebo-controlled Fatty Liver Improvement with Rosiglitazone Therapy (FLIRT) Trial. Gastroenterology 2008;135:100-110.
31. Ratziu V, Charlotte F, Bernhardt C, Giral P, Halbron M, Lenaour G, Hartmann-Heurtier A, et al. Long-term efficacy of rosiglitazone in

nonalcoholic steatohepatitis: results of the fatty liver improvement by rosiglitazone therapy (FLIRT 2) extension trial. Hepatology 2010;51:445-453.

32. Younossi Z, Ratziu V, Loomba R, Rinella M, Anstee QM, Goodman Z, Bedossa P, et al. Positive results from REGENERATE: A phase 3 international, randomized, placebo-controlled study evaluating obeticholic acid treatment for NASH. J Hepatol 2019;70:e5.
33. Alkhouri N, Dixon LJ, Feldstein AE. Lipotoxicity in nonalcoholic fatty liver disease: not all lipids are created equal. Expert Rev Gastroenterol Hepatol 2009;3:445-451.
34. Yamaguchi K, Yang L, McCall S, Huang J, Yu XX, Pandey SK, Bhanot S, et al. Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with nonalcoholic steatohepatitis. Hepatology 2007;45:1366-1374.
35. Kumashiro N, Erion DM, Zhang D, Kahn M, Beddow SA, Chu X, Still CD, et al. Cellular mechanism of insulin resistance in nonalcoholic fatty liver disease. Proc Natl Acad Sci U S A 2011;108:16381-16385.
36. Xia JY, Holland WL, Kusminski CM, Sun K, Sharma AX, Pearson MJ, Sifuentes AJ, et al. Targeted induction of ceramide degradation leads to improved systemic metabolism and reduced hepatic steatosis. Cell Metab 2015;22:266-278.
37. Stefan N, Haring HU. The metabolically benign and malignant fatty liver. Diabetes 2011;60:2011-2017.

38. Patterson RE, Kalavalapalli S, Williams CM, Nautiyal M, Mathew JT, Martinez J, Reinhard MK, et al. Lipotoxicity in steatohepatitis occurs despite an increase in tricarboxylic acid cycle activity. Am J Physiol Endocrinol Metab 2016;310:E484-494.
39. Magkos F, Su X, Bradley D, Fabbrini E, Conte C, Eagon JC, Varela JE, et al. Intrahepatic diacylglycerol content is associated with hepatic insulin resistance in obese subjects. Gastroenterology 2012;142:1444-1446 e1442.
40. Ter Horst KW, Gilijamse PW, Versteeg RI, Ackermans MT, Nederveen AJ, la Fleur SE, Romijn JA, et al. Hepatic diacylglycerol-associated protein kinase cepsilon translocation links hepatic steatosis to hepatic insulin resistance in humans. Cell Rep 2017;19:1997-2004.
41. Pagadala M, Kasumov T, McCullough AJ, Zein NN, Kirwan JP. Role of ceramides in nonalcoholic fatty liver disease. Trends Endocrinol Metab 2012;23:365-371.
42. Apostolopoulou M, Gordillo R, Koliaki C, Gancheva S, Jelenik T, De Filippo E, Herder C, et al. Specific hepatic sphingolipids relate to insulin resistance, oxidative stress, and inflammation in nonalcoholic steatohepatitis. Diabetes Care 2018;41:1235-1243.
43. Kalavalapalli S, Bril F, Guingab J, Vergara A, Garrett TJ, Sunny NE, Cusi K. Impact of exenatide on mitochondrial lipid metabolism in mice with nonalcoholic steatohepatitis. J Endocrinol 2019;241:293-305.
44. Kalavalapalli S, Bril F, Koelmel JP, Abdo K, Guingab J, Andrews P, Li WY, et al. Pioglitazone improves hepatic mitochondrial function in a mouse model of

nonalcoholic steatohepatitis. Am J Physiol Endocrinol Metab 2018;315:E163-E173.

FIGURE LEGENDS

Figure 1. Histological response based on the degree of IHTG reduction. Histological response was defined as either resolution of NASH without worsening of fibrosis (* in Panels A and B) or 2-point improvement in the NAFLD activity score (NAS) from two different parameters without worsening of fibrosis (# in Panels C and D). p values represent Chi^2 or Fisher's exact test among all groups.

Figure 2. Histological response among patients with vs. without steatosis resolution treated with pioglitazone or placebo. Normalization defined as intrahepatic triglyceride [IHTG] content of less than 5.56%. Histological response was defined as either resolution of NASH without worsening of fibrosis (* in Panel A) or 2-point improvement in the NAFLD activity score (NAS) from two different parameters without worsening of fibrosis (# in Panel B). p values represent Chi^2 or Fisher's exact test .

Figure 3. Rate of improvement in lobular inflammation (Panels A and B), hepatocyte ballooning (panels C and D), and liver fibrosis (panels D and E) based on the amount of relative reduction of intrahepatic triglyceride (IHTG) content after follow-up. p values represent Chi^2 or Fisher's exact among all groups.

Figure 4. Changes in lobular inflammation, hepatocyte ballooning, and liver fibrosis scores (panels A, C, and E) and proportion of patients with

improvement in those parameters (panels B, D, and F) among patients with vs. without steatosis resolution. Normalization defined as intrahepatic triglyceride [IHTG] content of less than 5.56%. Grey: placebo. White: pioglitazone. p values represent t-Student's test (panels A, C, and E) or Chi^2 (panels B, D, and F).

Figure 5. Changes in plasma ALT and AST based on the presence or absence of steatosis resolution (i.e., change of intrahepatic triglyceride [IHTG] content to less than 5.56%) in patients receiving placebo (panel A) or pioglitazone (panel B). p values represent t-Student's test.

Figure 6. Sensitivity analyses. Results shown in panel A excluded 37 patients that participated in the 6-month trial. For panel B, all patients were combined regardless of their treatment arm (n=121). Histological response was defined as either *resolution of NASH without worsening of fibrosis or $^{\#}$2-point improvement in the NAFLD activity score (NAS) from two different parameters without worsening of fibrosis. p values represent Chi^2 or Fisher's exact test among all groups.

Table 1. Baseline characteristics based on relative changes in IHTG content after follow-up.

Relative changes in intrahepatic triglyceride (IHTG) content by ^{1}H-MRS	No change or increase in IHTG (n=18)	Reduction of 1-30% (n=21)	Reduction of 31-50% (n=28)	Reduction of 51-70% (n=24)	Reduction of >70% (n=30)	p value

Complete data was available for all patients (n=121), except total body fat by DEXA that was available in 118 patients. ^{1}H-MRS: proton magnetic resonance spectroscopy; T2DM: type 2 diabetes mellitus; HbA1c: glycated hemoglobin A1c. p values calculated by one-way ANOVA for numerical variables and Chi^{2} for categorical variables

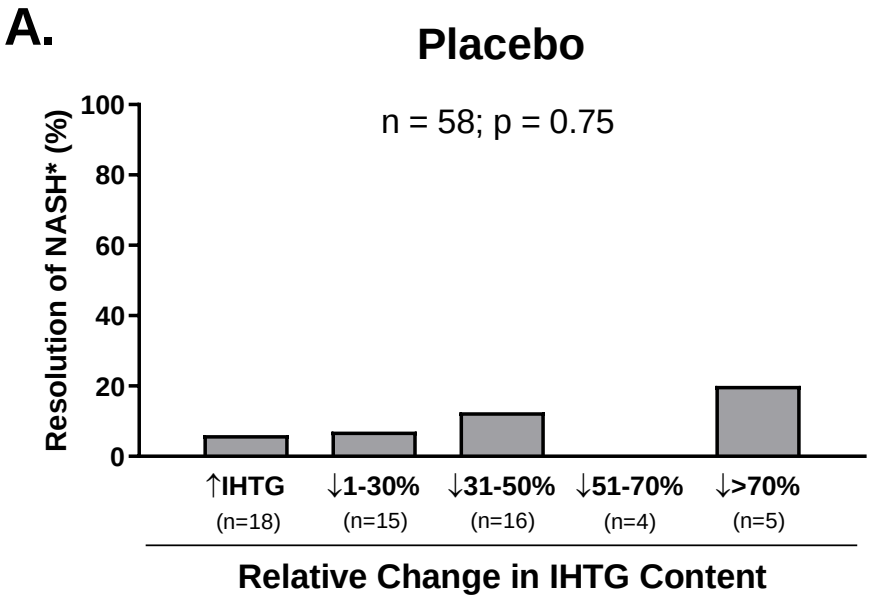
Patients on active drug (pioglitazone), %	0%	29%	43%	83%	83%	<0.001
Age, years	51 ± 9	52 ± 9	51 ± 11	54 ± 10	57 ± 7	0.11
Gender (male), %	67%	81%	68%	54%	67%	0.46
Body mass index, kg/m^2	32.6 ± 4.1	34.5 ± 4.5	34.0 ± 4.6	33.0 ± 4.8	34.1 ± 5.4	0.67
Total body fat, %	34 ± 9	33 ± 7	34 ± 8	35 ± 7	36 ± 8	0.77
Prevalence of T2DM, %	67%	67%%	50%	50%	70%	0.39
Fasting plasma glucose, mg/dl	124 ± 43	126 ± 33	130 ± 37	119 ± 25	138 ± 38	0.37
HbA1c, %	6.5 ± 1.4	6.6 ± 1.2	6.4 ± 1.3	6.2 ± 1.2	6.7 ± 1.0	0.52
Fasting plasma insulin, µIU/ml	14 ± 9	20 ± 14	16 ± 10	15 ± 9	11 ± 9	0.08
Total cholesterol, mg/dl	176 ± 34	192 ± 44	180 ± 39	178 ± 40	171 ± 46	0.51
LDL-C, mg/dl	107 ± 30	111 ± 40	116 ± 34	104 ± 36	100 ± 42	0.54
Triglycerides, mg/dl	148 (87–224)	145 (122–276)	136 (100–156)	160 (109–207)	143 (78–215)	0.38
HDL-C, mg/dl	37 ± 10	37 ± 9	36 ± 9	39 ± 10	40 ± 11	0.40
ALT, IU/ml	52 ± 37	63 ± 34	77 ± 41	52 ± 34	47 ± 29	0.014
AST, IU/ml	41 ± 20	46 ± 31	50 ± 22	45 ± 35	35 ± 17	0.25
Liver fat by ^{1}H-MRS, %	10 ± 5	13 ± 8	17 ± 10	15 ± 8	14 ± 7	0.05
NAFLD activity score (NAS)	4.6 ± 1.6	4.8 ± 1.8	4.4 ± 1.8	3.7 ± 1.9	3.8 ± 1.7	0.12
Steatosis grade	1.8 ± 0.9	2.0 ± 0.9	1.9 ± 0.8	1.6 ± 0.9	1.7 ± 0.9	0.56
Inflammation grade	1.5 ± 0.5	1.6 ± 0.6	1.5 ± 0.6	1.3 ± 0.6	1.3 ± 0.6	0.24
Ballooning grade	1.3 ± 0.7	1.2 ± 0.8	1.1 ± 0.8	0.9 ± 0.8	0.8 ± 0.7	0.06
Fibrosis stage	1.6 ± 1.1	1.3 ± 1.0	1.2 ± 1.1	1.2 ± 1.2	1.0 ± 1.0	0.43

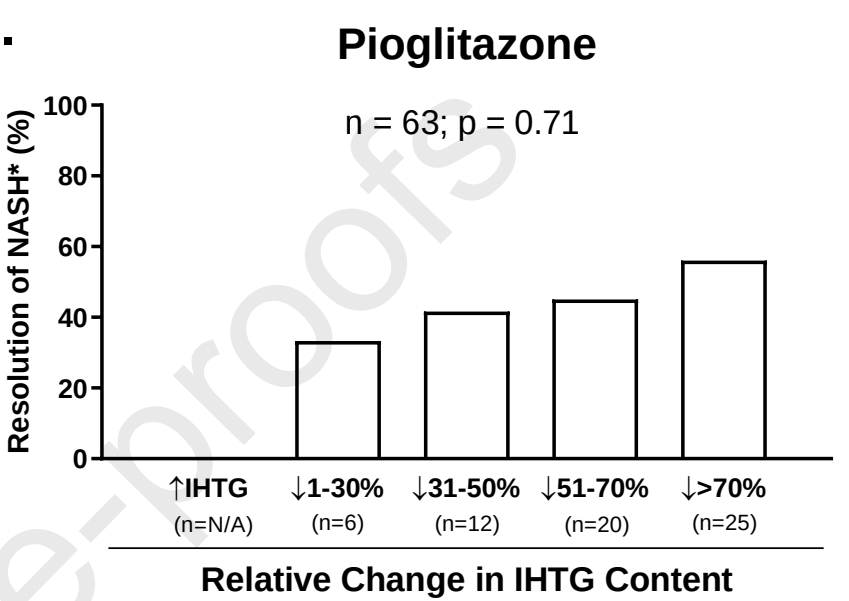
Table 2. Prediction of resolution of NASH without worsening of fibrosis based on changes in IHTG content in all patients combined (placebo and pioglitazone).

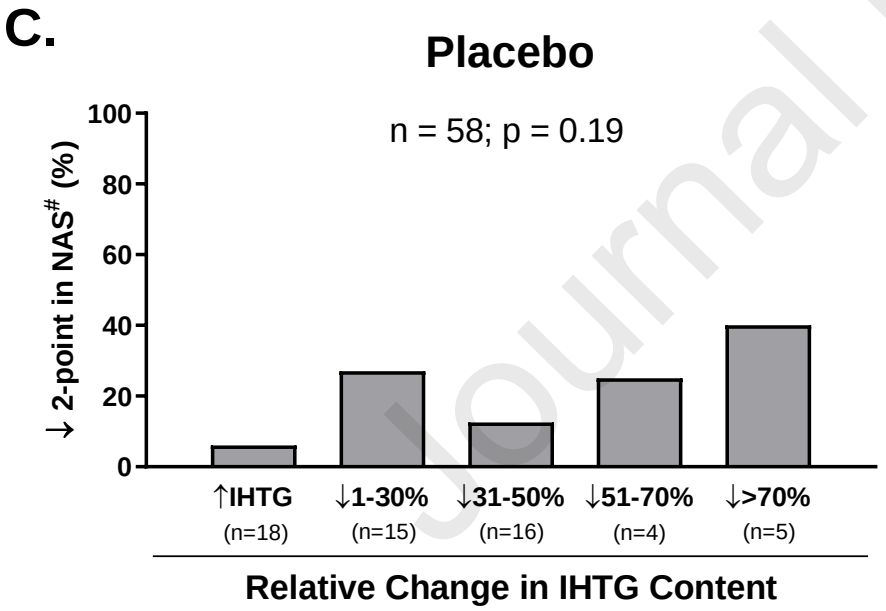
Relative changes in intrahepatic triglyceride (IHTG) content by ^{1}H-MRS	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Reduction of **10%** or more	**94%**	**26%**	**34%**	**92%**
Reduction of **15%** or more	**94%**	**30%**	**36%**	**93%**
Reduction of **20%** or more	**94%**	**35%**	**37%**	**94%**
Reduction of **25%** or more	**91%**	**38%**	**38%**	**92%**
Reduction of **30%** or more	**89%**	**41%**	**38%**	**90%**
Reduction of **35%** or more	**86%**	**47%**	**40%**	**90%**
Reduction of **40%** or more	**86%**	**52%**	**42%**	**90%**
Reduction of **45%** or more	**80%**	**59%**	**44%**	**88%**
Reduction of **50%** or more	**69%**	**65%**	**44%**	**84%**
Reduction of **55%** or more	**66%**	**70%**	**47%**	**83%**
Reduction of **60%** or more	**63%**	**78%**	**54%**	**84%**
Reduction of **65%** or more	**49%**	**83%**	**53%**	**80%**
Reduction of **70%** or more	**43%**	**83%**	**50%**	**78%**
Reduction of **75%** or more	**37%**	**86%**	**52%**	**77%**
Reduction of **80%** or more	**23%**	**93%**	**57%**	**75%**
Reduction of **85%** or more	**17%**	**94%**	**54%**	**74%**
Reduction of **90%** or more	**11%**	**98%**	**67%**	**73%**

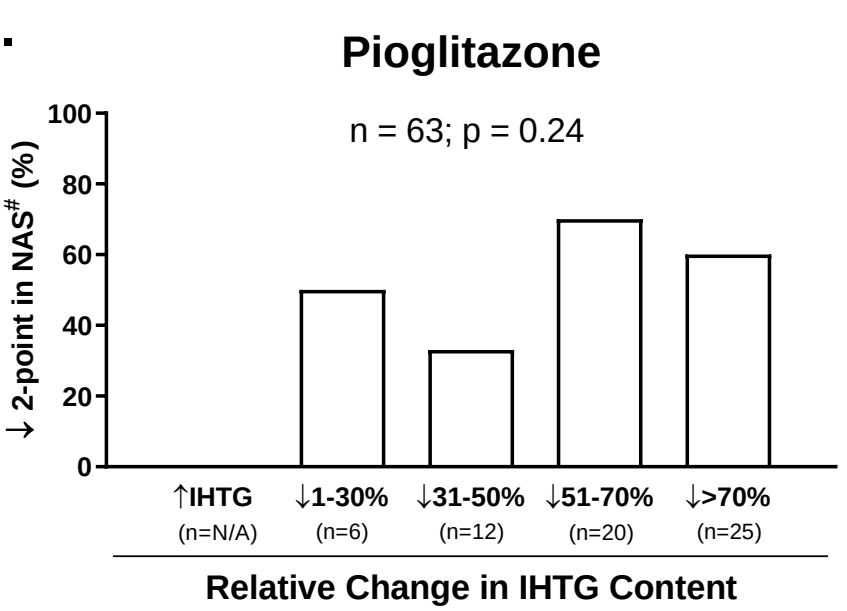
Complete data available for all patients (n=121). ^{1}H-MRS: proton magnetic resonance spectroscopy; PPV: positive predictive value; NPV: negative predictive value. Sensitivity, specificity, PPV, and NPV calculated for predicting resolution of NASH without worsening of fibrosis.

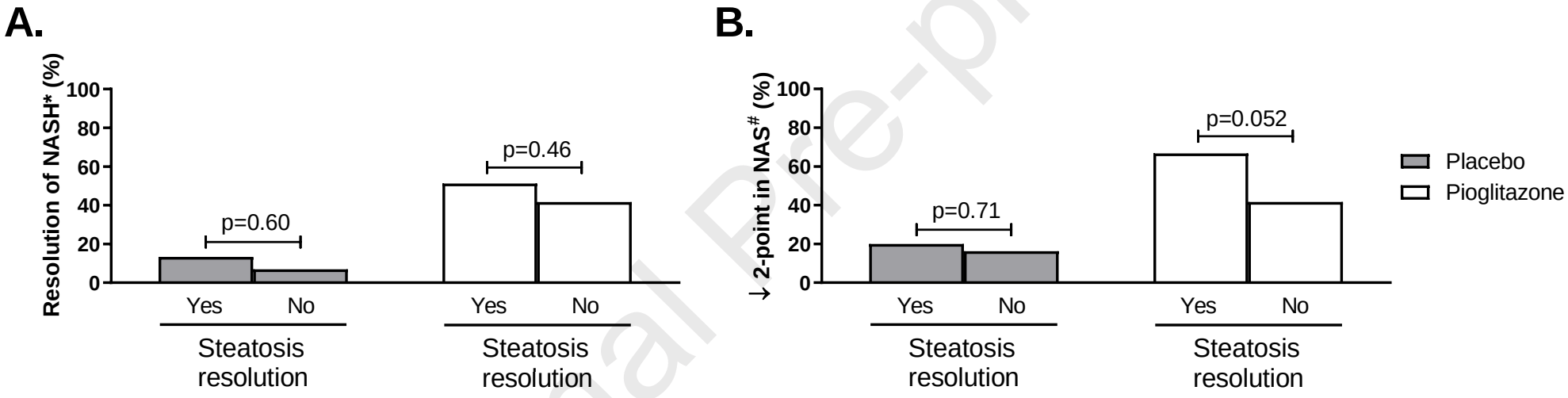
- Magnetic resonance-based images are frequently used in phase 1-2 studies for NASH.
- It is assumed that changes in liver fat will predict histological changes.
- We analyzed data from 121 patients with paired biopsies and magnetic resonance imaging.
- Changes in liver fat did not predict changes in inflammation, ballooning or fibrosis.
- Quantification of liver fat after treatment may be misleading as a surrogate marker of treatment response.

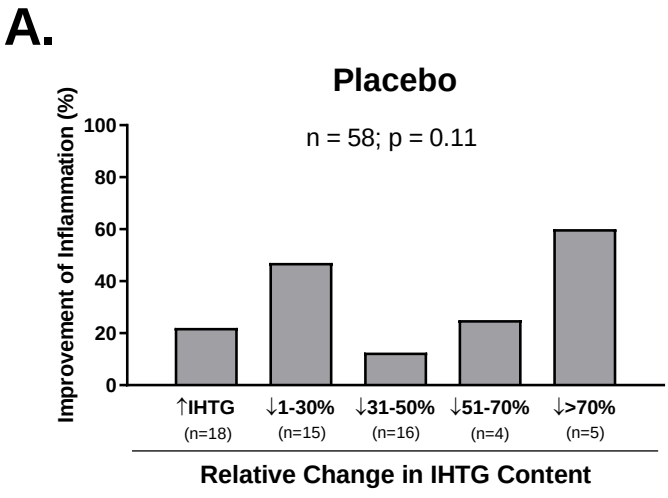


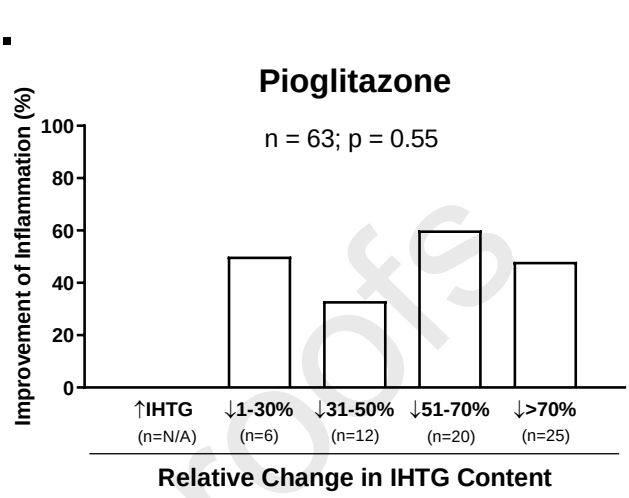


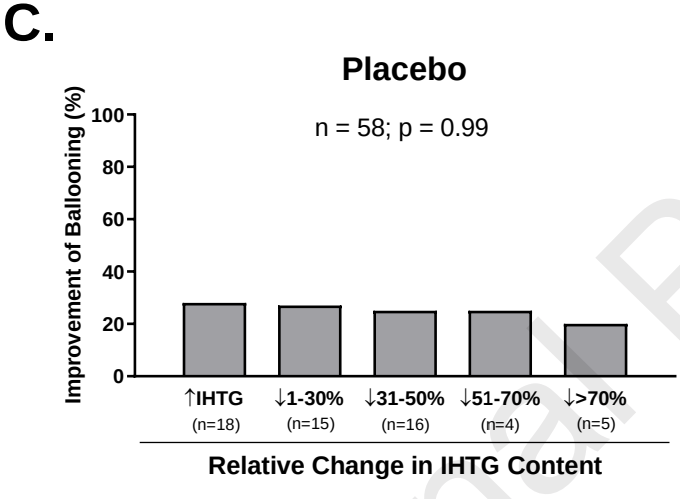


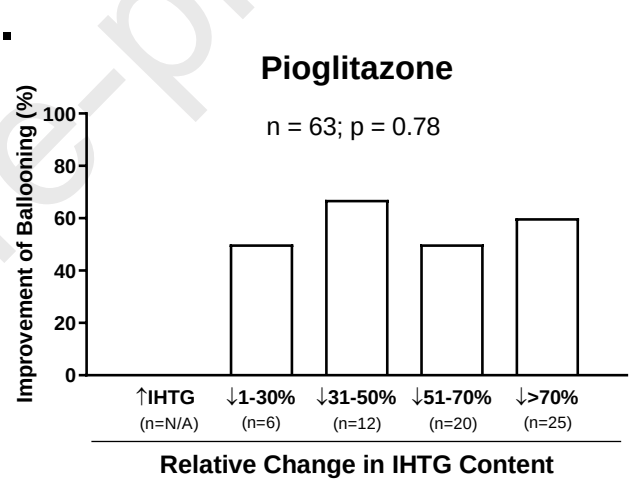


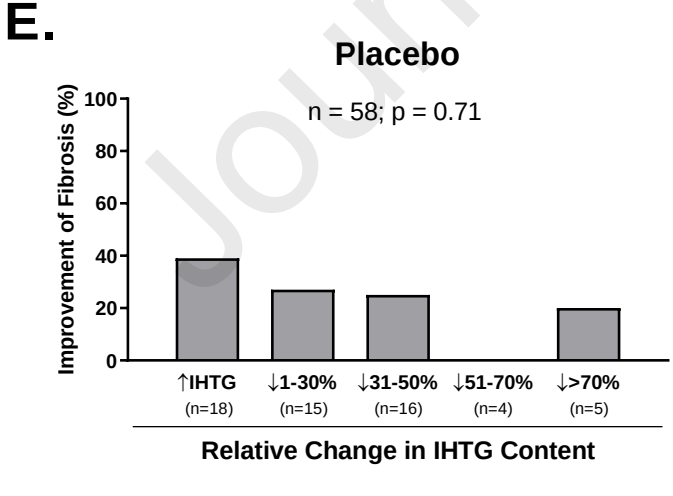


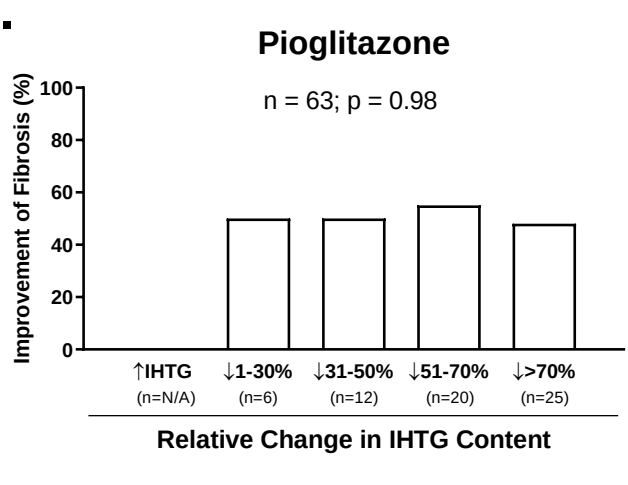


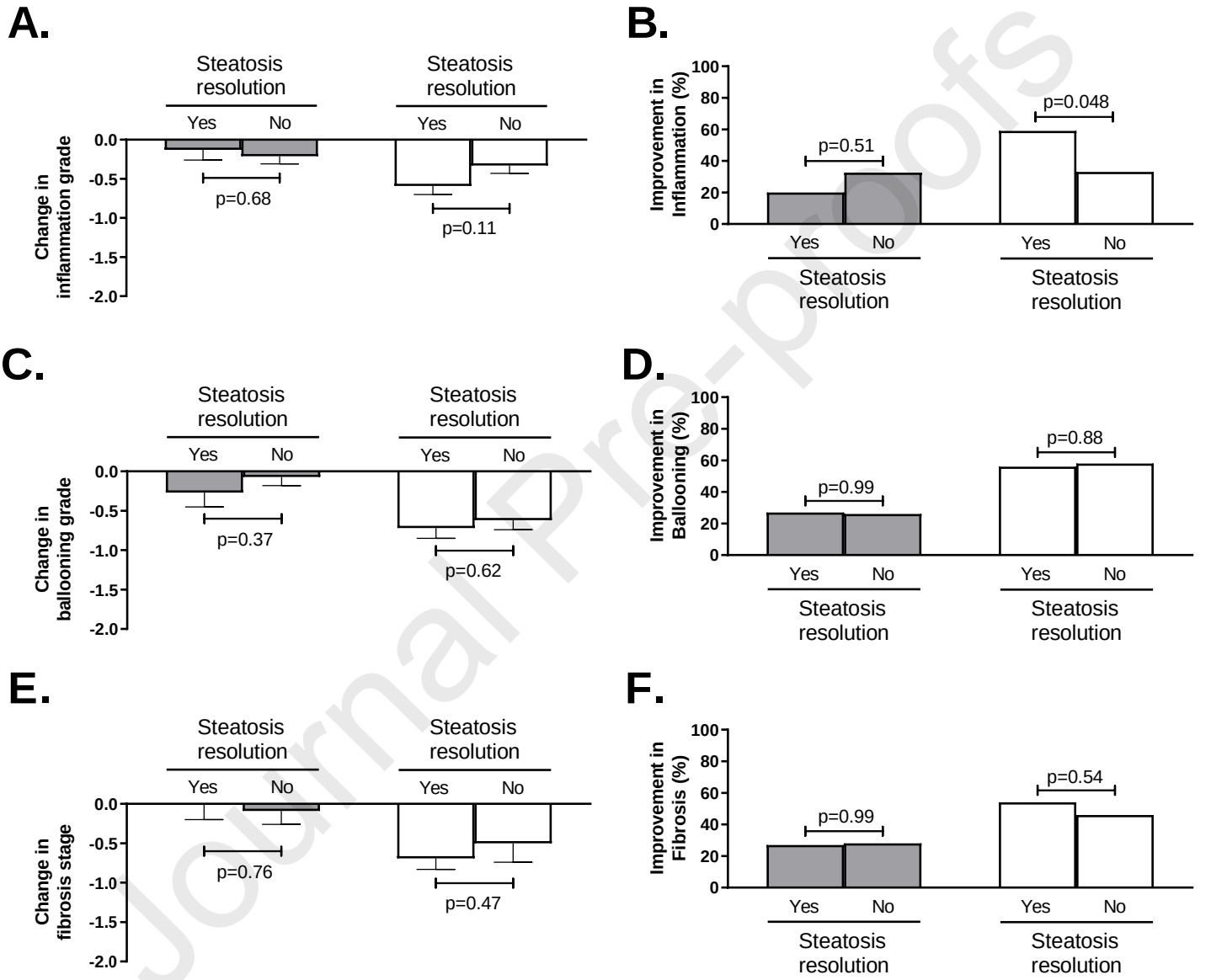


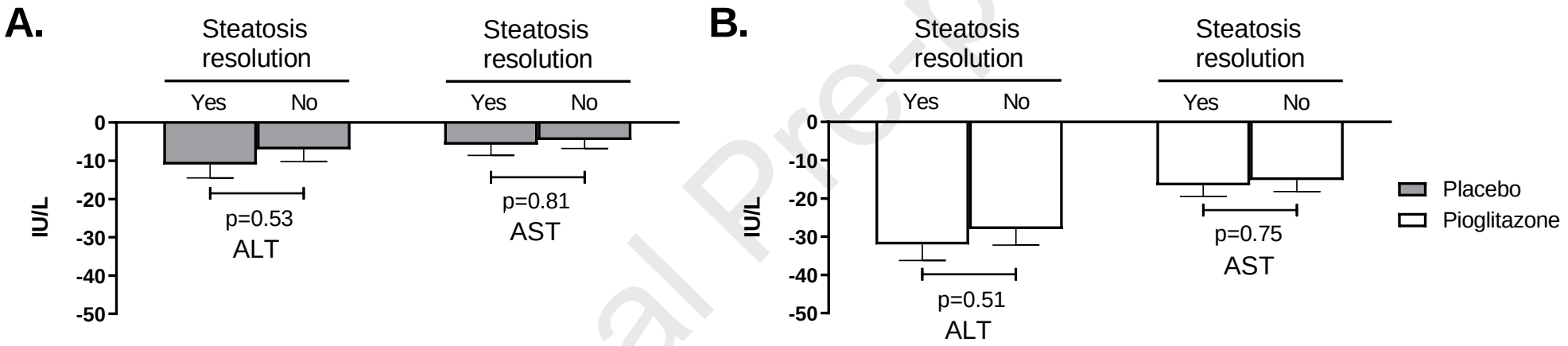











A.
Steatosis resolution
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No
Steatosis resolution
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-30
-40
-50
IU/L
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ALT
p=0.81
AST
B.
Steatosis resolution
Yes
No
Steatosis resolution
Yes
No
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ALT
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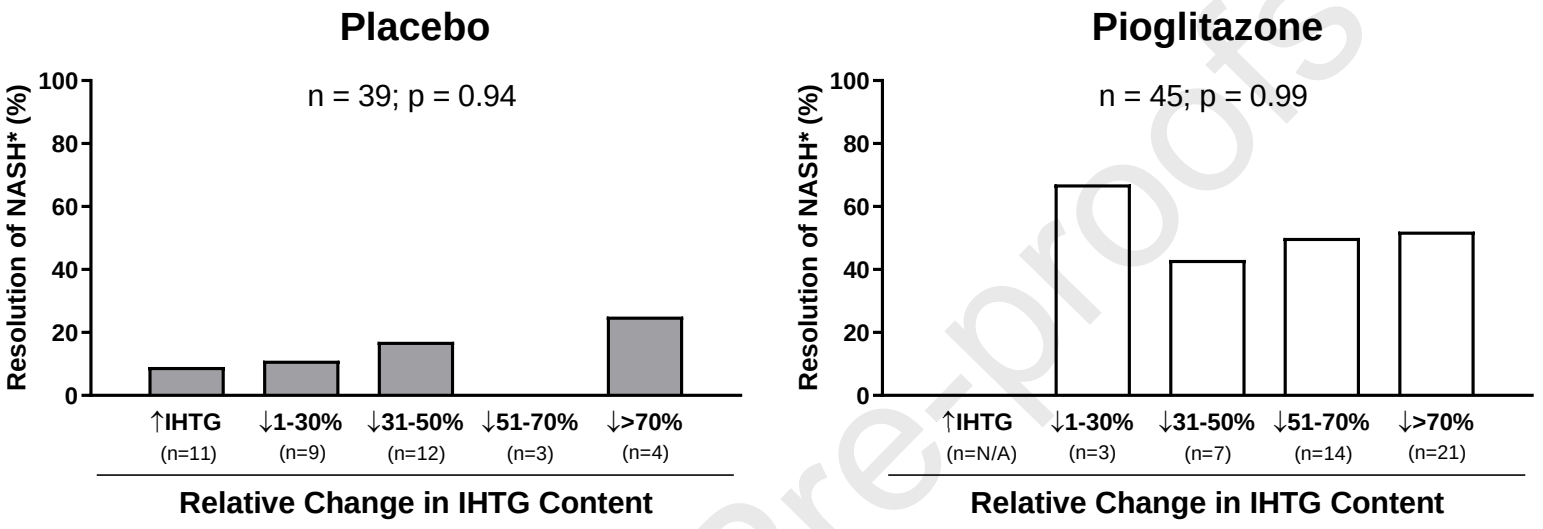
AST
Placebo
Pioglitazone

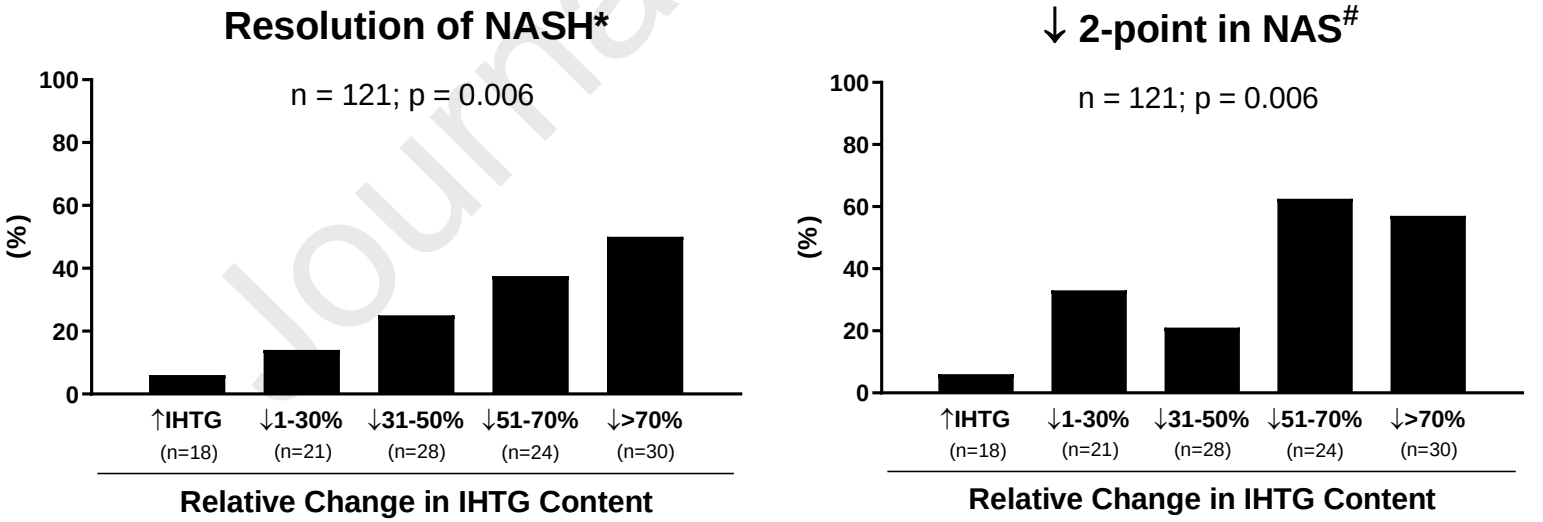
A.

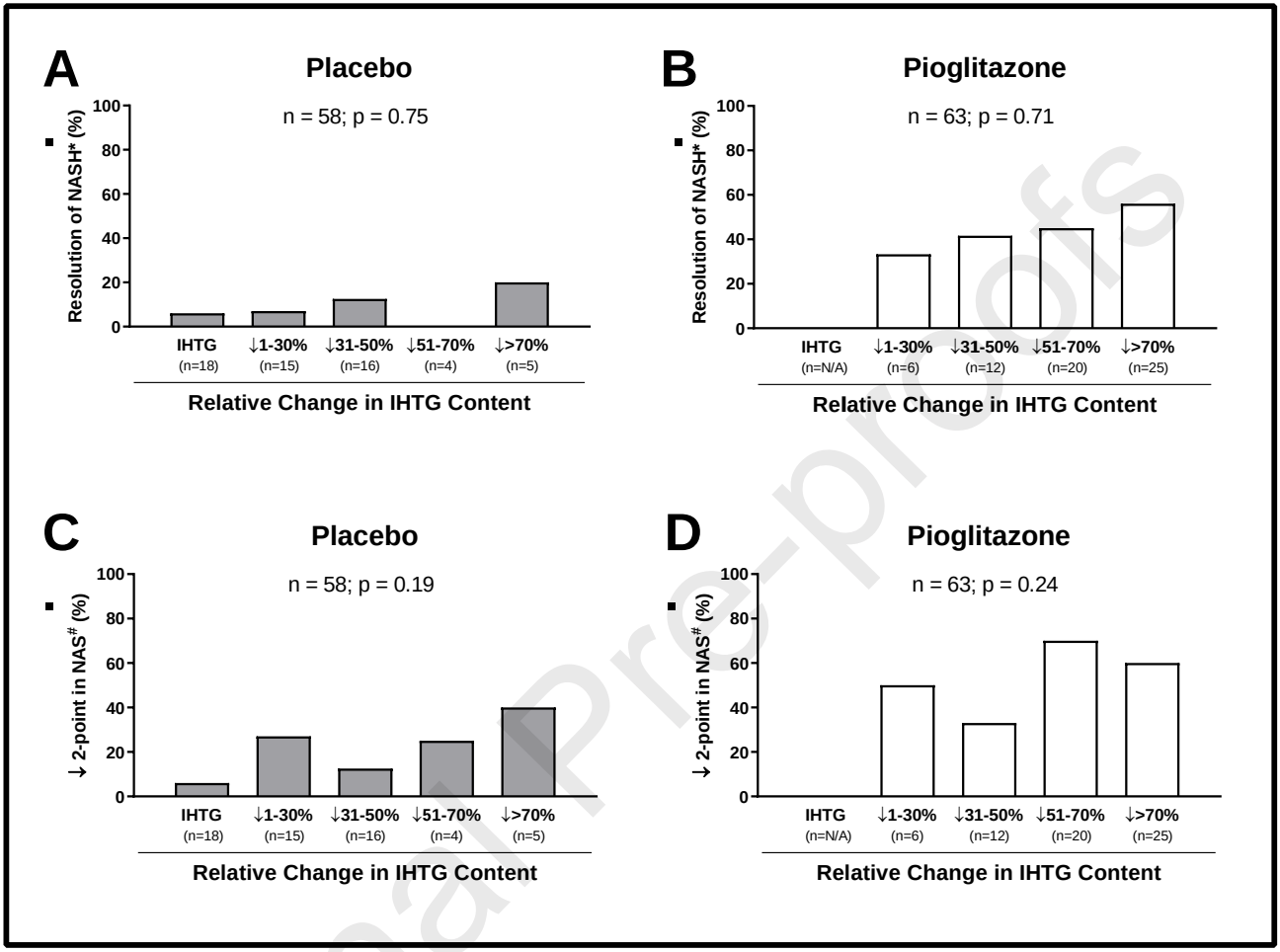
Sensitivity Analysis: excluding patients from the 6-month trial



B.

Sensitivity Analysis: combining patients on pioglitazone and placebo





Suggests that:

Changes in Intrahepatic Triglyceride Content

Do NOT predict

Changes in Histological Parameters (i.e., Inflammation, Ballooning, Fibrosis)