

The Postbiotic ReFerm® Versus Standard Nutritional Support in Advanced Alcohol-Related Liver Disease (GALA-POSTBIO):

A randomized controlled phase 2 trial

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Supplementary Material

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65 **Supplementary tables and figures:**

66 Supplementary tables:

| | Total | ReFerm® | Fresubin® | p-value |
|---|------------------|------------------|------------------|---------|
| PEth, baseline | 0.20 (0.00-1.00) | 0.16 (0.00-0.89) | 0.20 (0.00-1.28) | 0.57 |
| PEth, 4 weeks | 0.11 (0.00-1.03) | 0.23 (0.00-1.03) | 0.11 (0.00-1.21) | 0.68 |
| PEth, 24 weeks | 0.45 (0.00-1.46) | 0.31 (0.02-1.46) | 0.70 (0.00-1.51) | 0.88 |
| Supplementary Table 1: Comparing median (IQR) PEth values between the intervention groups through the trial. Two-sided P-values was derived using Wilcoxon rank-sum test. | | | | |

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| | ReFerm® | | Fresubin® | |
|--|-------------------|------------------------|-------------------|------------------------|
| | Responders n=8 | Non-responders n=13 | Responders n=4 | Non-responders n=15 |
| PEth <0.05, n | 2 | 2 | 2 | 5 |
| PEth >0.05, n | 6 | 11 | 2 | 10 |
| Supplementary Table 2: PEth measurements in responders vs. non-responders. Subgroup analysis of number of participants achieving the primary endpoint (≥10% reduction in α-SMA expression) according to PEth values at baseline as indicators for alcohol abstinence at time of inclusion. “Responders” are defined as patients who meet the primary endpoint of. | | | | |

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| | Total n= 56 | ReFerm® n=28 | Fresubin® n=28 | p-value |
|---|----------------|-----------------|-------------------|---------|
| Any adverse events, n(%) | 44 (78%) | 24(86%) | 20(71%) | 0.190 |
| Increased satiety, n(%) | 27 (50%) | 16(59%) | 11(41%) | 0.170 |
| Bloated, n(%) | 19 (38%) | 11 (42%) | 8 (33%) | 0.510 |
| Borborygmi, n(%) | 22 (41%) | 10 (37%) | 12 (44%) | 0.580 |
| Nausea, n(%) | 5 (9%) | 2 (7%) | 3 (11%) | 0.640 |
| Self reported weight loss, n(%) | 6 (11%) | 4 (15%) | 2 (7%) | 0.390 |
| Feeling of weakness/muscle fatigue, n(%) | 4 (7%) | 2 (7%) | 2 (7%) | 1.000 |
| Dizziness, n(%) | 8 (14%) | 5 (19%) | 3 (11%) | 0.440 |
| Abnormal taste sensation in the mouth, n(%) | 6 (11%) | 5 (19%) | 1 (4%) | 0.083 |
| Bad breath, n(%) | 6 (11%) | 4 (15%) | 2 (7%) | 0.039 |
| Hunger, n(%) | 11 (20%) | 6 (22%) | 5 (19%) | 0.740 |
| Other, n(%) | 17 (31%) | 9 (33%) | 8 (30%) | 0.770 |

Supplementary Table 3: List of adverse events. Data are shown as number of events with incidences in parenthesis. P-values are derived using Pearson’s chi-square test.
The most common adverse events were increased satiety, borborygmi, and bloating with no difference between groups. If patients encountered mild but intolerable side effects, their daily intake was reduced by half. Two patients withdrew from the study due to bloating, which persisted despite dosage reduction. Another patient could tolerate 50% of the daily dose due to bloating.

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| Reported in the current manuscript | Plan to be reported later |
|--|--|
| Primary | Secondary |
| 10% or more reduction in α -SMA expression in immunohistochemically stained liver biopsies | <ul style="list-style-type: none">- Reduction in circulating α-SMA concentration- Hepatic venous pressure gradient (HVPG)- Forns index- APRI score- FIB4- cytokeratin-18 degradation products M30 and M65- Liver vein outflow of microbial products- Metabolic changes- Ultra sonographic steatosis assessment- Collagen proportionate area (%) |
| Secondary | |
| Liver biopsies: <ul style="list-style-type: none">- Hepatic α-SMA expression- Kleiner fibrosis stage- Lobular inflammation- Hepatic steatosis- Hepatocyte ballooning | |
| Non-invasive: <ul style="list-style-type: none">- Transient liver elastography (TE)- Fibrosis-4 index (FIB-4)- N-terminal pro-peptide of type III collagen (PRO-C3)- Transient elastography (TE)- Shear Wave Elastography (SWE)- Enhanced Liver Fibrosis test (ELF test)- Controlled Attenuation Parameter (CAP) | |
| Omic features: <ul style="list-style-type: none">- Taxonomy- Gut microbial richness- Hepatic inflammation markers- Metabolites- Aminoacid- Short-chain fatty acids- Lipidomics | |
| Supplementary Table 4: Complete list of outcomes according to protocol | |

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| | ReFerm® n=7 | Fresubin® n=8 |
|--|------------------|------------------|
| Age (IQR), years | 56 (44-63) | 62 (52-65) |
| Male sex, n | 6 (86%) | 7 (88%) |
| Body mass index ≥ 30 kg/m ² , n | 3 (43%) | 3 (38%) |
| Type 2 diabetes, n | 1 (14%) | 1 (12%) |
| Alcohol consumption | | |
| Abstinent \geq one week prior to inclusion, n | 5 (71%) | 5 (62%) |
| Abstinent \geq one year prior to inclusion, n‡ | 2 (29%) | 3 (38%) |
| Average alcohol intake for active drinkers (IQR), gram/day | 69 (0-69) | 5 (5-7) |
| Phosphatidylethanol (IQR), μ /L‡ | 0.00 (0.00-1.00) | 0.02 (0.00-2.11) |
| Laboratory data | | |
| Alanintransaminase (IQR), U/L | 32 (21-56) | 28 (24-34) |
| Aspartattransaminase (IQR) U/L | 46 (25-81) | 36 (24-40) |
| Gamma-Glutamyltransferase (IQR), U/L | 163 (38-629) | 117 (45-269) |
| Bilirubin, (IQR), μ mol/L | 14 (9-17) | 9 (6-31) |
| Plateles (SD), 10 ⁹ /L | 167 (86) | 156 (36) |
| INR (SD) | 1.15 (0.17) | 1.11 (0.21) |
| Albumin (SD), g/L | 42 (3) | 42 (3) |
| C-reaktivt protein (IQR), mg/L | 3 (1-5) | 2 (1-6) |
| MELD-Na (IQR) | 9 (8-10) | 9 (9-12) |
| Child-Pugh class A | 7 (100%) | 8 (100%) |
| Liver histology | | |
| Kleiner fibrosis stage 2/3/4 | 1/1/3 | 1/1/4 |
| Lobular inflammation grade 0/1/2/3 | 2/2/1/0 | 1/4/1/0 |
| Ballooning grade 0/1/2 | 4/1/0 | 2/4/0 |
| Steatosis grade 0/1/2/3 | 4/0/1/0 | 1/3/2/0 |
| Non-invasive test of liver disease | | |
| Liver stiffness, measured by transient elastography (IQR), kPa | 47.0 (22.9-52.1) | 19.1 (13.3-23.4) |
| Controlled attenuation parameter (SD), dB/m | 270.7 (78.1) | 281.1 (55.7) |
| Shear wave elastography (SD), kPa | 28 (12) | 21 (11) |
| PRO-C3 (SD), ng/ml | 31.51 (14.75) | 24.78 (5.10) |
| Enhanced liver fibrosis test (SD)* | 11.1 (1.1) | 10.8 (0.7) |
| Fibrosis-4 score (IQR)* | 3.2 (1.4-4.3) | 2.2 (1.7-4.0) |

Supplementary Table 6: Demographic and baseline clinical characteristics of patients that did not conclude the study. Normally distributed data are presented as mean (SD), non-normally distributed data as median (IQR), and categorical data as counts (proportion, %). The sum of percentages may deviate from 100 due to rounding. INR, international normalised ratio; MELD-Na, model for end-stage liver disease sodium; α -SMA, α smooth muscle actin activation, PROC3; fragment of N-terminal type III collagen.

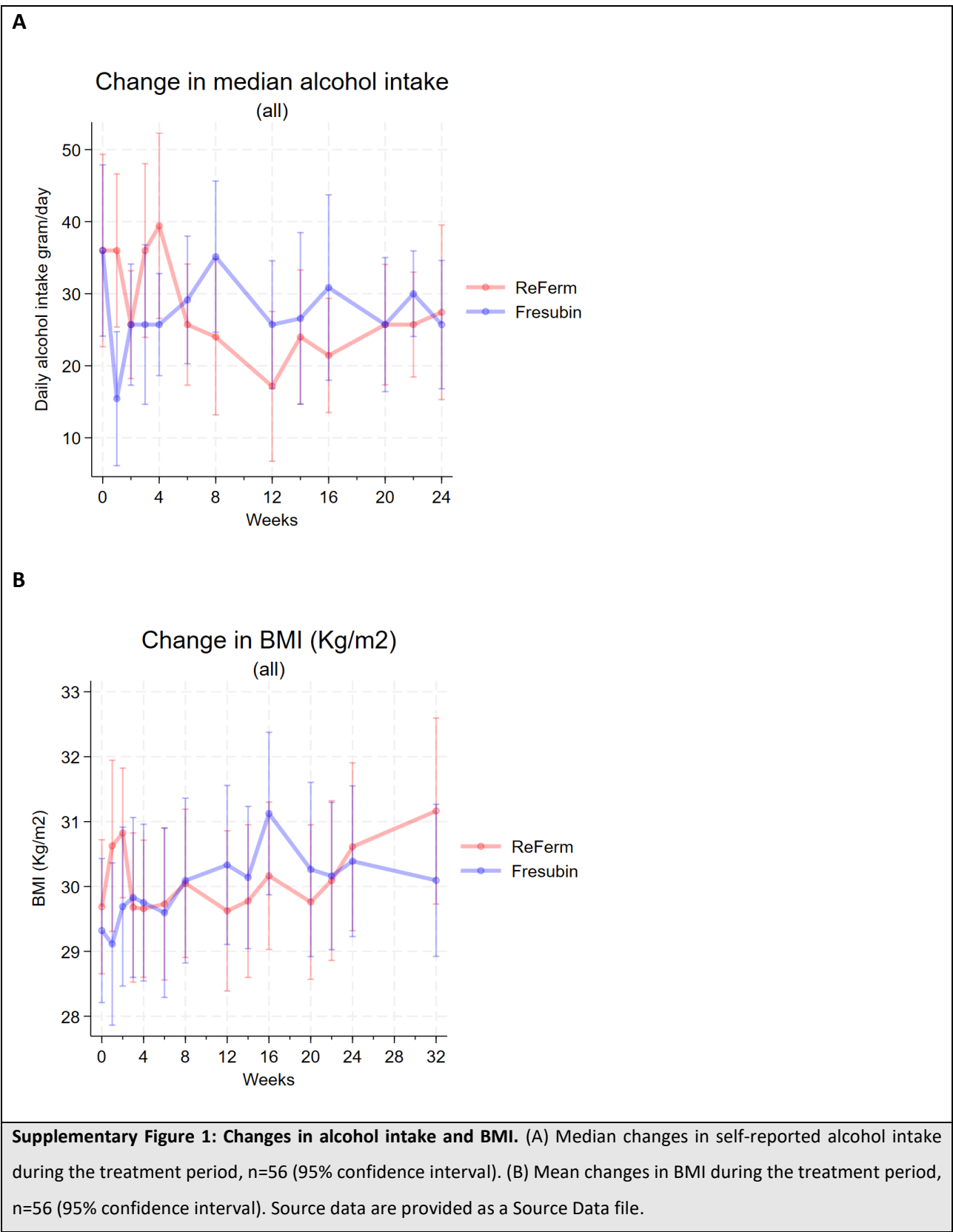
| Ingredients | Control Diet (V1534–300) |
|---|-----------------------------|
| Crude protein, % | 19.0 |
| Crude fat, % | 3.3 |
| Crude fibers, % | 4.9 |
| Crude ash, % | 6.4 |
| Starch, % | 35.2 |
| Sugar, % | 5.3 |
| Vitamin A, IU/kg chow | 25,000 |
| Vitamin D ₃ , IU/kg chow | 1,500 |
| Vitamin E, mg/kg chow | 125 |
| Vitamin K ₃ , mg/kg chow | 20 |
| Copper, mg/kg chow | 5 |
| Supplementary Table 7: Animal Chow. Manufacturer: Ssniff Spezialdiäten, Soest, Germany; IU, international units. | |

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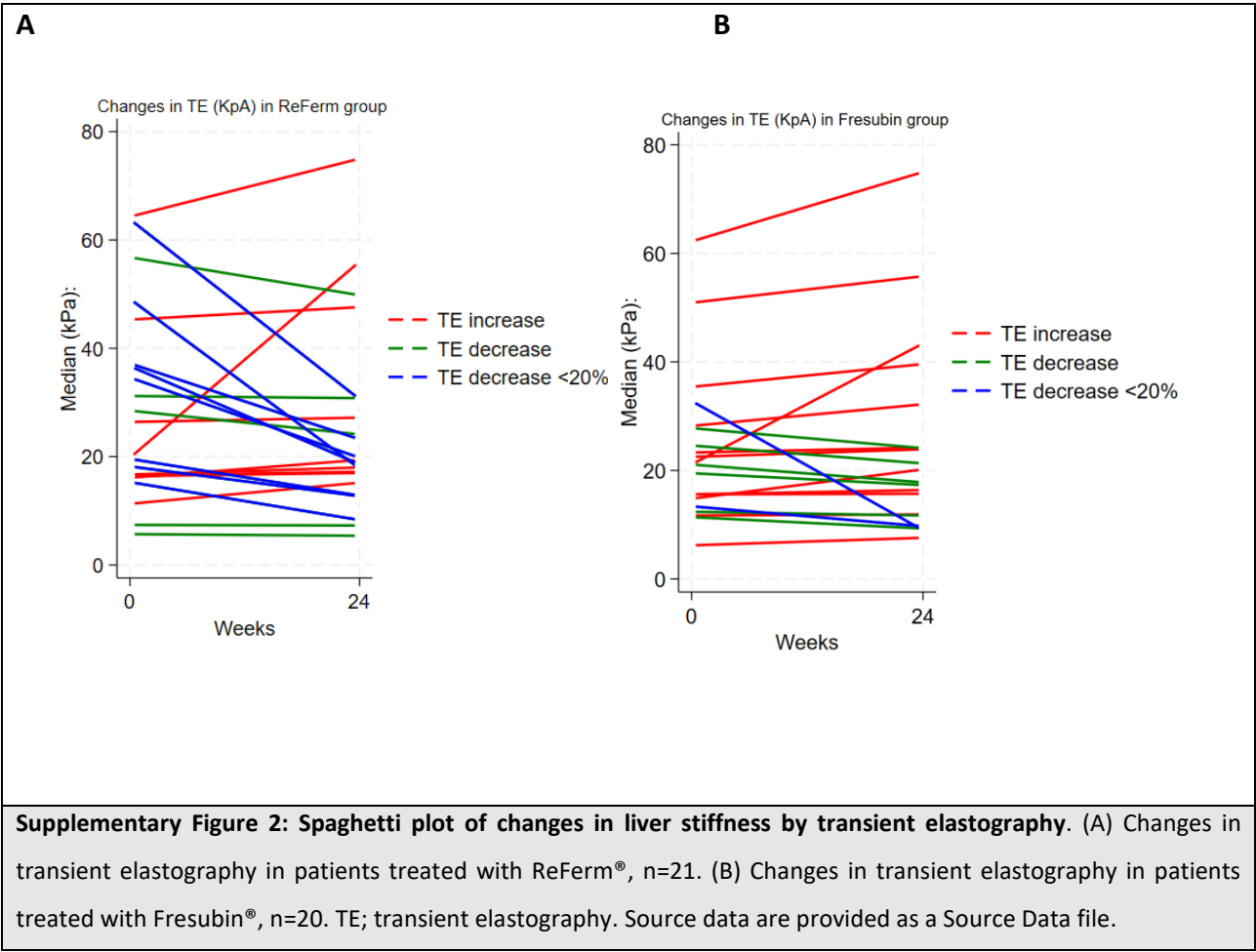
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| Gene | Assay ID | Species |
|---|---------------|--------------|
| Col1a1 | Mm00801666_g1 | Mus musculus |
| Acta2 (α -SMA) | Mm00725412_s1 | Mus musculus |
| Vinculin | Mm00447745_m1 | Mus musculus |
| Supplementary Table 8: Gene expression assays. Manufacturer: Thermo Fisher Scientific. Col1a1, collagen type 1a1; Acta2 (α -SMA), α -smooth muscle actin. | | |

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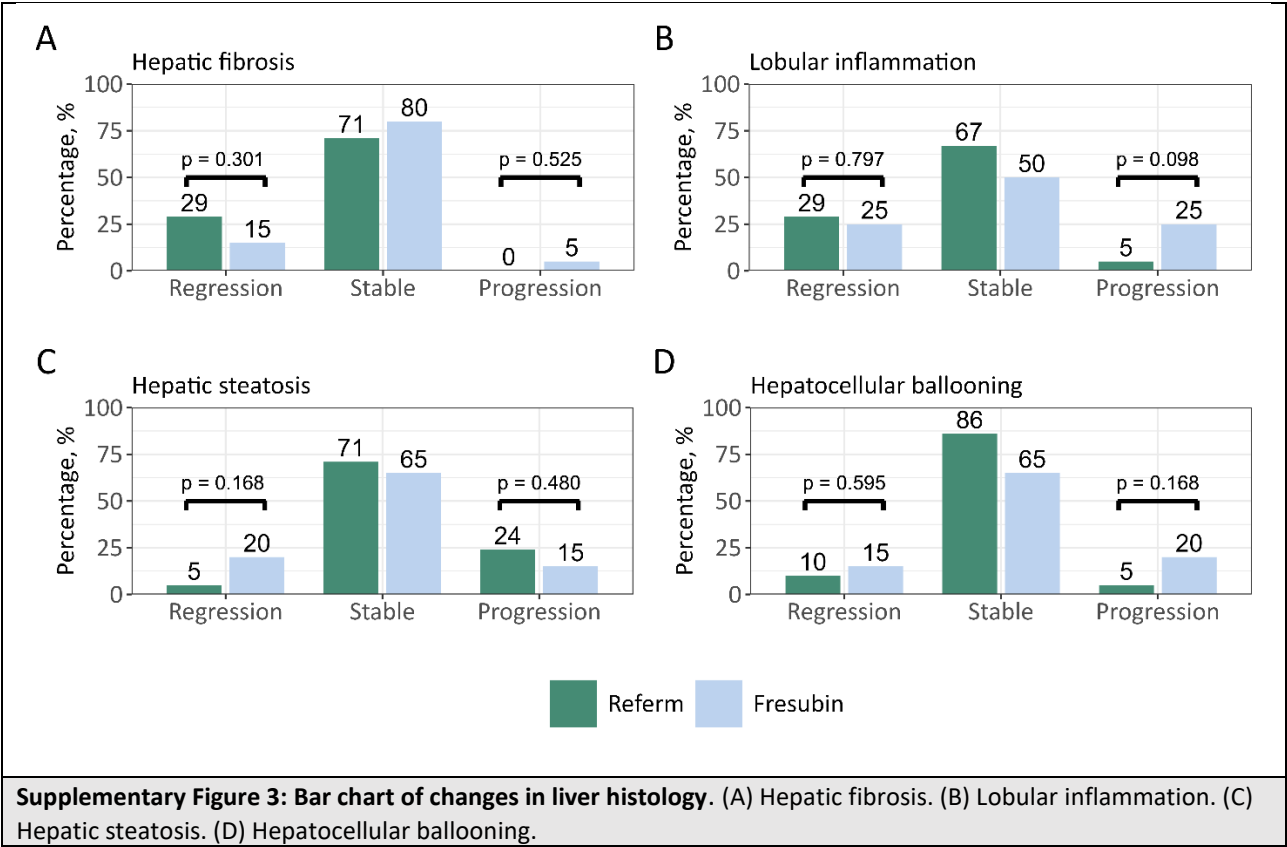


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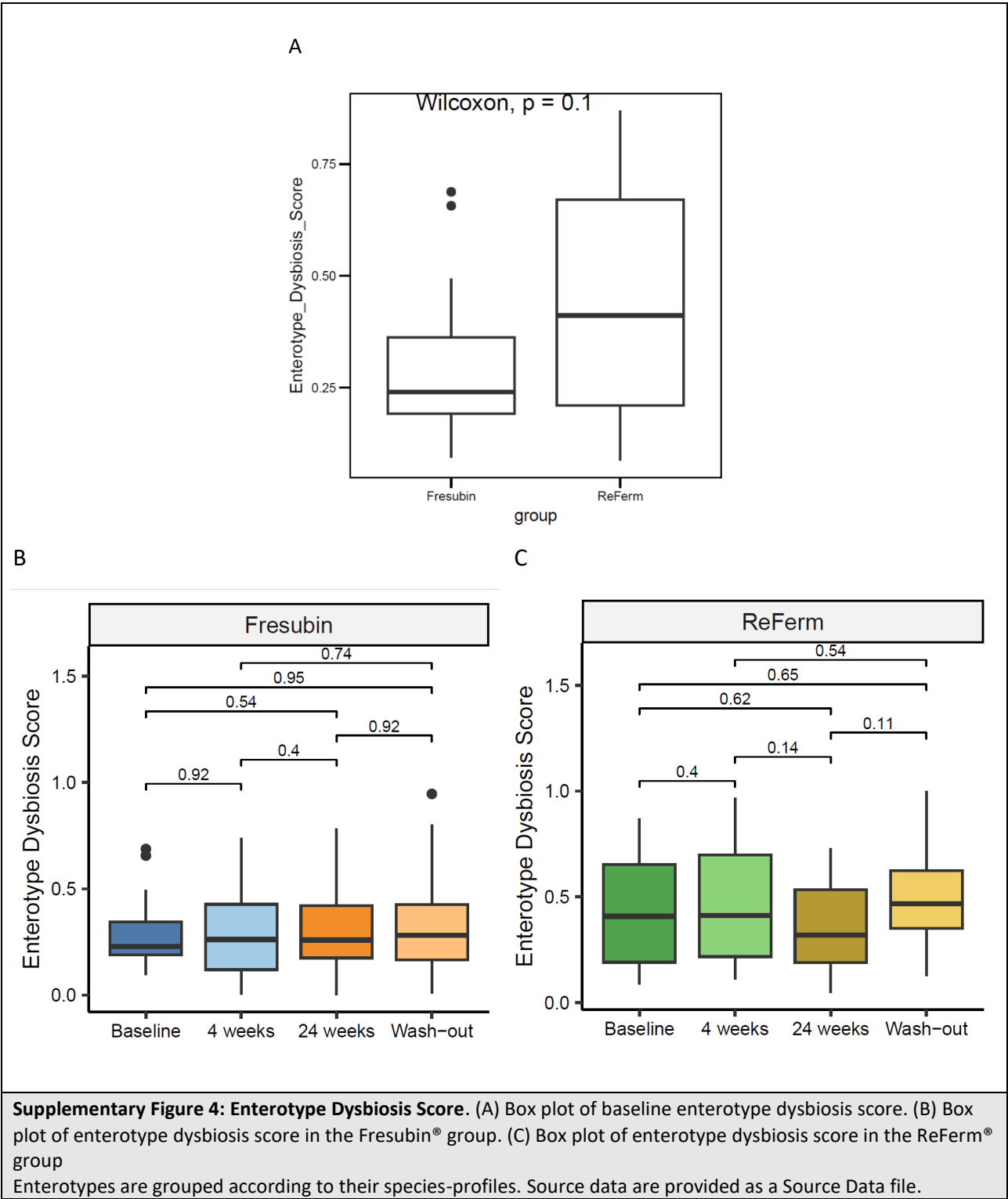
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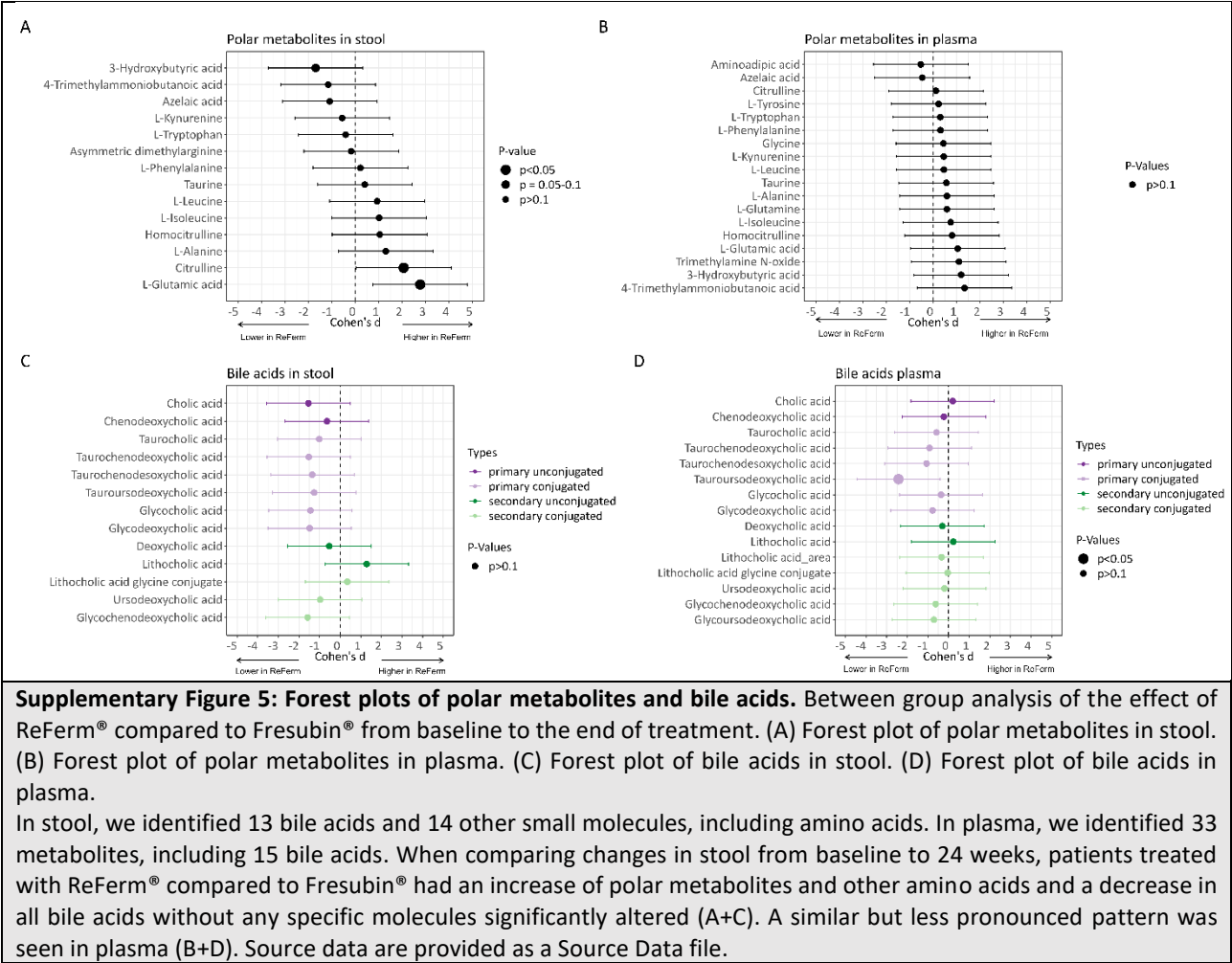
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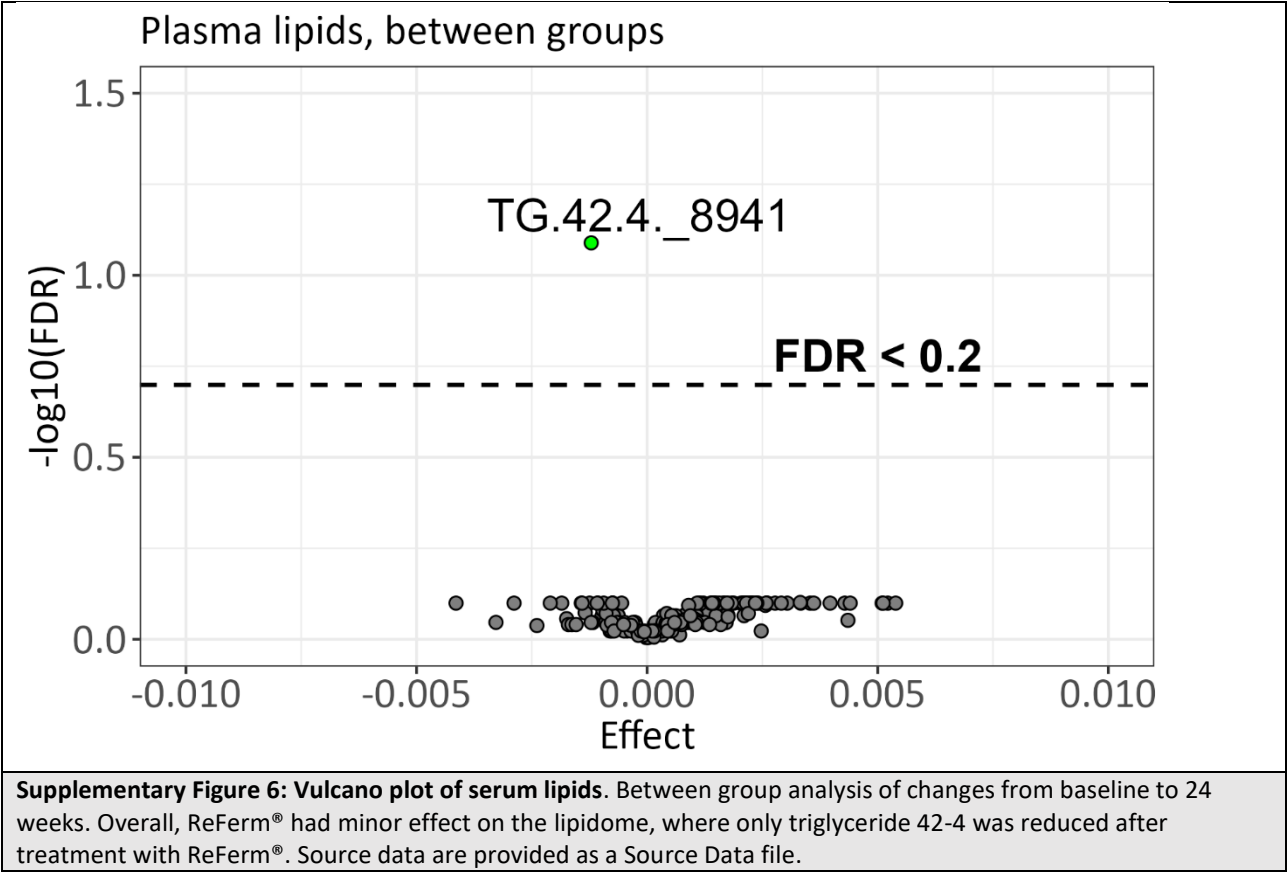
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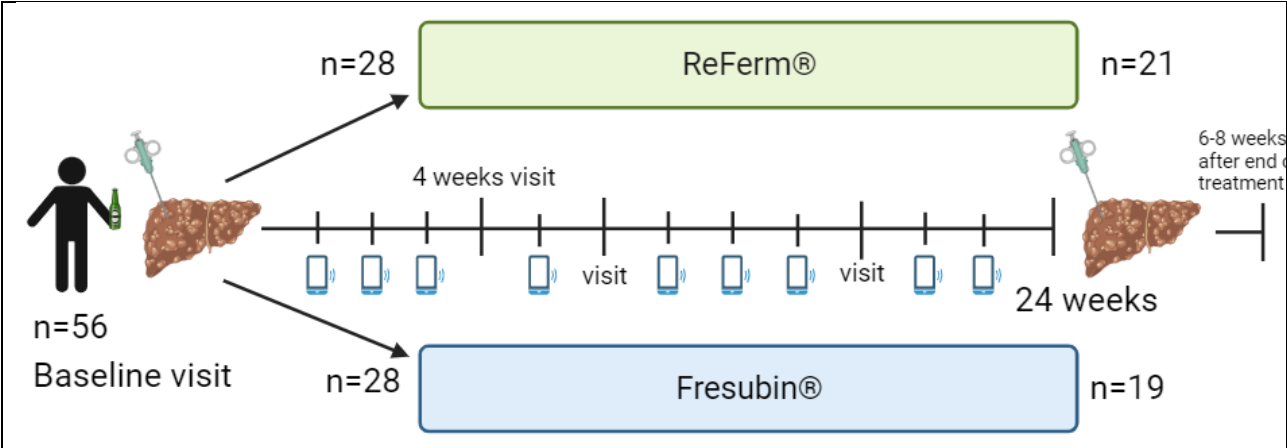
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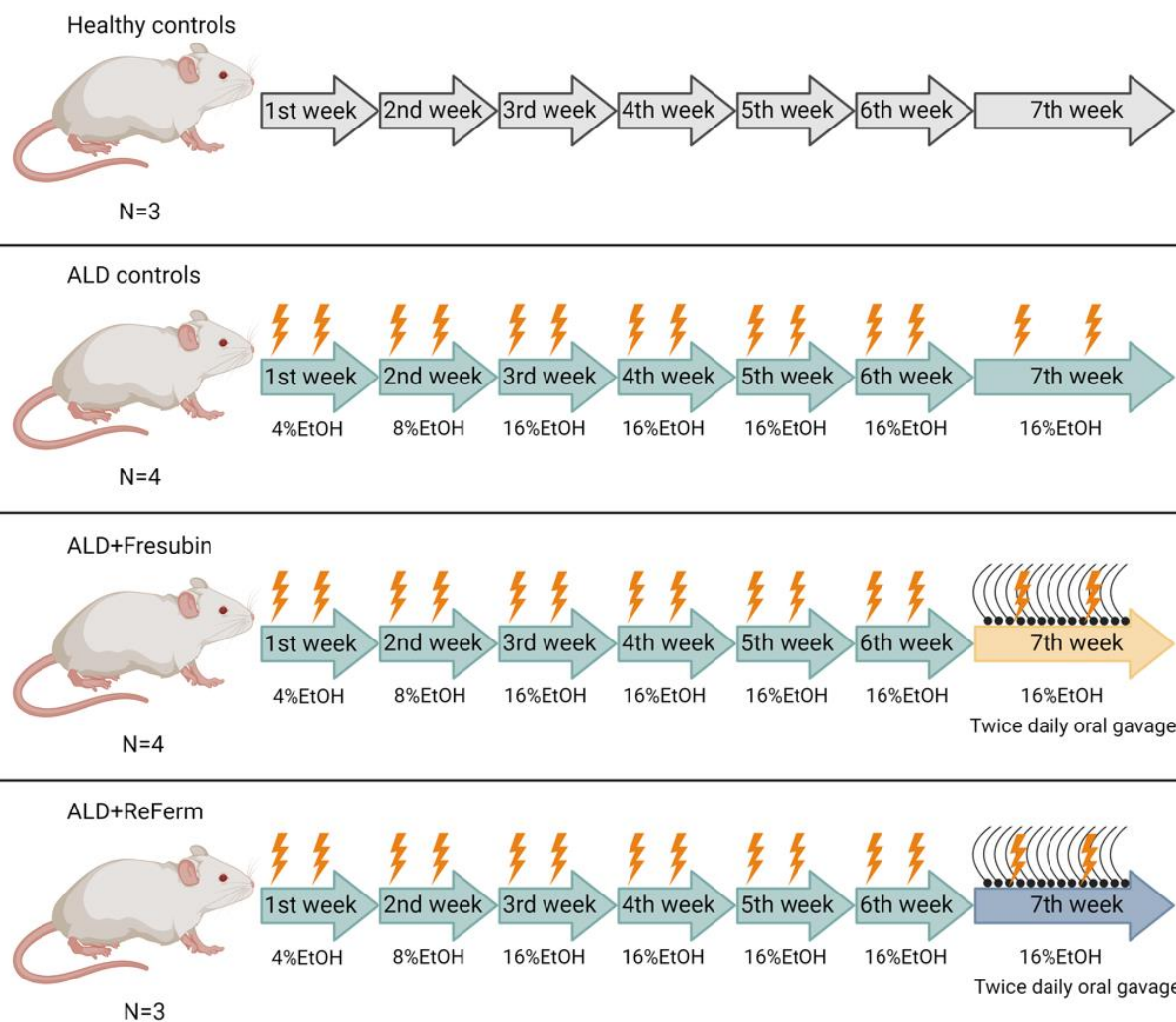


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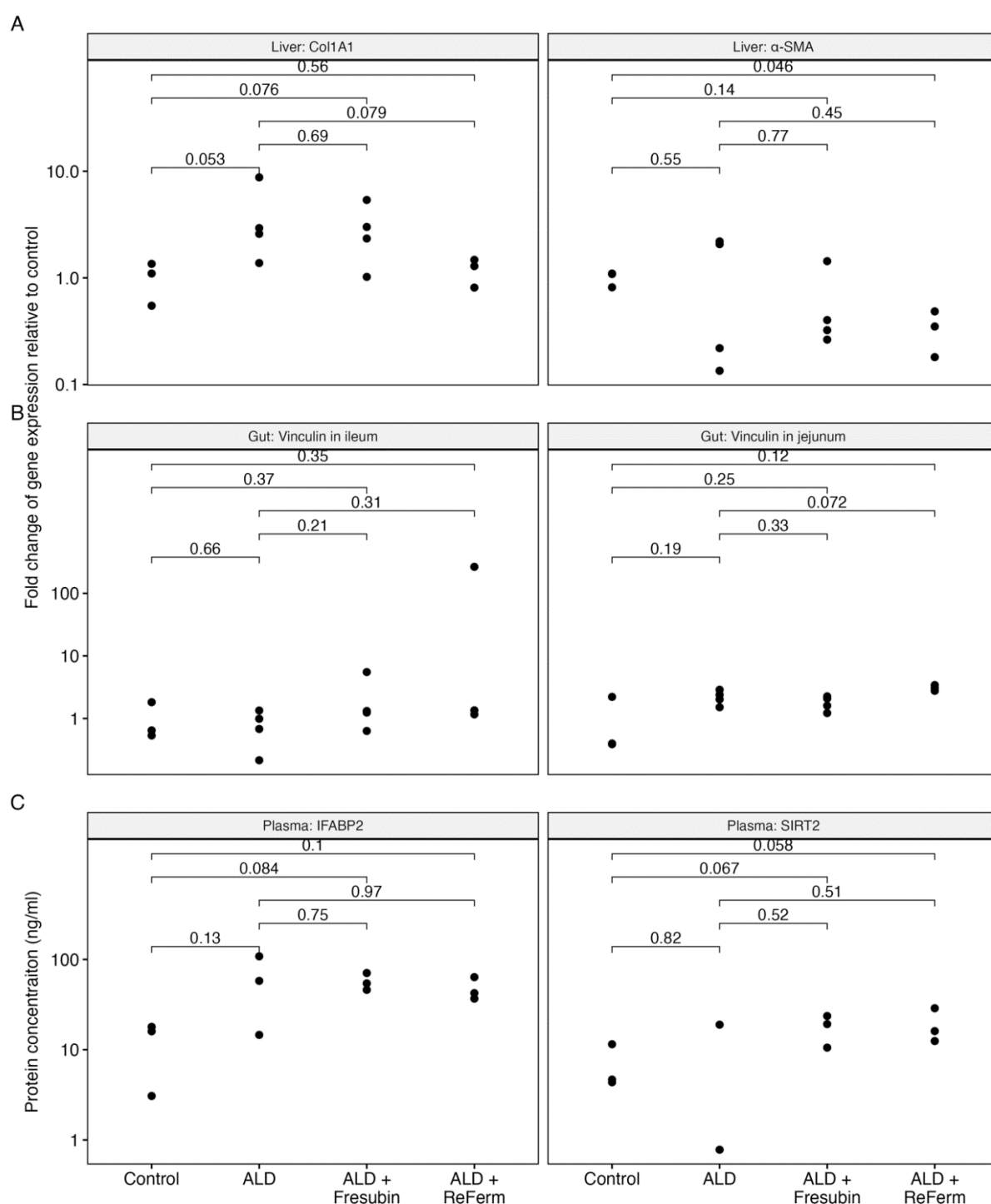
Supplementary Figure 7: Outline of the study design. At each in-hospital visit, patients brought the lids from their consumed study products for assessment of compliance and further reported the consumed amount of product. In case of discrepancies, the patient was confronted with this and, in collaboration with the investigator, a final compliance measure was reported. In the phone call assessments, patients reported the consumed amount.



Supplementary Figure 8: Outline of the animal study design. 11 specific pathogen-free male wild-type mice, aged 12 weeks had induced alcohol-related liver disease (2x/week CCl₄:2μl/g). The intervention groups received twice daily oral gavage (0.3 ml) of Fresubin® or ReFerm® for one week prior to sacrifice. Age-matched, untreated mice served as controls for all experiments. Created in <https://BioRender.com>

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Supplementary Figure 9: Bar charts of changes in animal models. A) Hepatic gene expression levels. B) mRNA of vinculin in ileum and jejunum. C) ELISA I-FABP and SIRT2 in plasma. P-values were calculated using t-tests on the log-transformed data.

Col1A1: Collagen 1, α -SMA: α -smooth muscle actin, I-FABP: Intestinal fatty acid-binding protein, SIRT2: NAD-dependent protein deacetylase sirtuin-2. Source data are provided as a Source Data file.