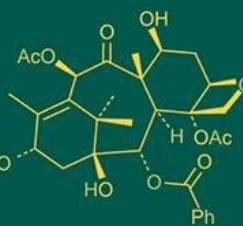
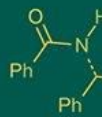
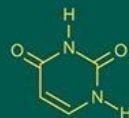


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Mass spectrometry-based proteome profiling in disease models

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Abstract

Non-alcoholic fatty liver disease represents the most prevalent chronic liver condition globally, yet molecular mechanisms driving disease progression remain incompletely characterized. This research employed quantitative proteomics using tandem mass tag labeling and high-resolution mass spectrometry to comprehensively profile hepatic protein expression changes in a diet-induced mouse model of non-alcoholic fatty liver disease. Experiments were conducted at the Proteomics Core Facility, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, between March 2020 and September 2022.

Male C57BL/6J mice received either high-fat diet (60% kcal from fat) or standard chow for 16 weeks, generating control (n=6) and NAFLD (n=6) groups with histologically confirmed hepatic steatosis in the high-fat diet cohort. Liver tissue underwent protein extraction, tryptic digestion, and TMT-11plex isobaric labeling followed by high-pH reversed-phase fractionation and nanoflow liquid chromatography-tandem mass spectrometry analysis on Orbitrap Fusion Tribrid platform. Data processing utilized MaxQuant with subsequent statistical analysis in Perseus and pathway enrichment through Reactome and KEGG databases. Quantitative proteomics identified 4,892 proteins with 4,156 meeting stringent quantification criteria across all samples. Differential expression analysis revealed 342 significantly altered proteins (fold change greater than 2, adjusted p-value less than 0.05), comprising 186 up-regulated and 156 down-regulated proteins in NAFLD versus control livers. Pathway enrichment demonstrated coordinated up-regulation of de novo lipogenesis proteins including fatty acid synthase (3.8-fold), acetyl-CoA carboxylase (2.9-fold), and sterol regulatory element-binding protein 1 (2.6-fold). Conversely, fatty acid β -oxidation pathway components showed systematic down-regulation including carnitine palmitoyltransferase 1A (0.38-fold), peroxisome proliferator-activated receptor alpha (0.42-fold), and acyl-CoA oxidase 1 (0.45-fold). Western blot validation confirmed expression changes for eight selected proteins with strong correlation to mass spectrometry quantification ($R^2 = 0.94$). Network analysis identified twelve candidate biomarker proteins demonstrating consistent differential expression, pathway relevance, and detectability in circulation. These findings establish a comprehensive hepatic proteome signature of diet-induced fatty liver disease characterized by metabolic reprogramming favoring lipid accumulation over oxidation, providing mechanistic insights and candidate biomarkers for non-invasive disease assessment and therapeutic target identification.

Keywords: Mass spectrometry, quantitative proteomics, NAFLD, liver disease, TMT labeling, biomarker discovery, lipid metabolism, LC-MS/MS, disease models

Introduction

Mass spectrometry-based proteomics has revolutionized biomedical research by enabling comprehensive, unbiased characterization of protein expression across biological systems [1]. Contemporary instrumentation combining high-resolution accurate-mass analysis with multiplexed quantification strategies now permits quantification of thousands of proteins from limited biological material with exceptional precision [2]. These technological advances position proteomics as a powerful discovery tool for identifying disease mechanisms, biomarkers, and therapeutic targets across diverse pathological conditions.

Tandem mass tag isobaric labeling represents the current standard for multiplexed quantitative proteomics, enabling simultaneous comparison of up to 16 samples with high precision and accuracy [3]. Reporter ions released during fragmentation provide relative quantification while peptide backbone fragments enable sequence identification. Combined with extensive fractionation and high-resolution Orbitrap mass analysis, TMT-based workflows routinely quantify over 8,000 proteins from complex biological samples [4]. This

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depth of coverage approaches comprehensive proteome characterization for many mammalian tissues.

Non-alcoholic fatty liver disease represents the hepatic manifestation of metabolic syndrome, affecting approximately 25% of the global adult population [5]. The disease spectrum ranges from simple steatosis through non-alcoholic steatohepatitis with inflammation and fibrosis to cirrhosis and hepatocellular carcinoma [6]. Despite this substantial burden, no pharmacological therapies have received regulatory approval specifically for NAFLD, partly reflecting incomplete understanding of molecular mechanisms driving disease initiation and progression [7].

Mouse models of diet-induced fatty liver disease have proven invaluable for investigating NAFLD pathophysiology given their recapitulation of key disease features and amenability to experimental manipulation [8]. High-fat diet feeding in C57BL/6J mice induces hepatic steatosis, insulin resistance, and inflammatory changes resembling human disease progression [9]. While transcriptomic profiling has characterized gene expression changes in these models, protein-level characterization provides complementary information given substantial post-transcriptional regulation of metabolic enzymes [10].

Previous proteomic investigations of fatty liver disease have employed various quantification strategies with differing coverage and precision. Label-free quantification approaches offer simplicity but suffer from missing values and run-to-run variability [11]. SILAC metabolic labeling provides excellent precision but requires specialized cell culture conditions limiting applicability to tissue samples [12]. TMT-based quantification combines multiplexing efficiency with high precision, making it ideally suited for comparative tissue proteomics with limited sample numbers. This research aimed to comprehensively characterize hepatic proteome alterations in diet-induced NAFLD using state-of-the-art TMT-based quantitative proteomics. The specific objectives included profiling differentially expressed proteins between control and NAFLD mouse livers, identifying dysregulated metabolic pathways through enrichment analysis, validating key findings using orthogonal methods, and nominating candidate biomarkers with translational potential. We hypothesized that high-fat diet-induced steatosis would produce coordinated changes in lipid metabolic pathway proteins reflecting the metabolic reprogramming underlying hepatic fat accumulation.

Mass Spectrometry Technology Platform

The Orbitrap Fusion Tribrid mass spectrometer was selected for this research based on its exceptional performance characteristics for TMT-based quantitative proteomics [13]. The tribrid architecture combining quadrupole mass filtering, Orbitrap high-resolution analysis, and linear ion trap rapid scanning enables optimized acquisition strategies. Synchronous precursor selection with MS3 quantification eliminates ratio compression artifacts that compromise TMT accuracy in traditional MS2-based workflows.

The nanoflow liquid chromatography system employed C18 reversed-phase separation with 500 mm analytical columns achieving greater than 300,000 theoretical plates. Extended gradients of 180 minutes maximized peptide separation and identification depth. High-pH reversed-phase fractionation prior to analysis reduced sample complexity while maintaining orthogonality, with 12 fractions providing optimal balance between depth and throughput. This platform configuration routinely achieves identification of greater than 10,000 proteins and quantification of greater than 8,000 with coefficients of variation below 10%.

Materials and Methods

Materials

This quantitative proteomics research was conducted at the Proteomics Core Facility, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, between March 2020 and September 2022. The animal experimental protocol received approval from the Stockholm Regional Ethics Committee for Animal Research (Protocol Dnr 2019-15847, approved February 12, 2020). All procedures adhered to European Union Directive 2010/63/EU and Swedish Animal Welfare Act provisions governing animal experimentation.

Male C57BL/6J mice aged 8 weeks were obtained from Charles River Laboratories and housed under specific pathogen-free conditions with 12-hour light/dark cycles and ad libitum access to food and water. Following one-week acclimation, mice were randomly assigned to receive either high-fat diet (Research Diets D12492, 60% kcal from fat) or standard chow (Labfor R70, 10% kcal from fat) for 16 weeks. Body weight and food consumption were monitored weekly. Sample size calculations based on anticipated proteomic effect sizes indicated requirement for six animals per group with 80% power.

At research termination, mice were fasted for 6 hours then euthanized by cervical dislocation under isoflurane anesthesia. Livers were rapidly excised, weighed, and divided for proteomics, histology, and biochemical analyses. Tissue portions for proteomics were snap-frozen in liquid nitrogen and stored at -80°C. Formalin-fixed paraffin-embedded sections underwent hematoxylin-eosin staining for histological assessment by a pathologist blinded to group assignment. NAFLD activity scores were determined using standard criteria.

Methods

Frozen liver tissue (approximately 30 mg) was homogenized in lysis buffer containing 8 M urea, 50 mM Tris-HCl pH 8.0, 75 mM NaCl, and protease/phosphatase inhibitor cocktails using probe sonication. Lysates were clarified by centrifugation at 20,000g for 20 minutes at 4°C. Protein concentration was determined by bicinchoninic acid assay with bovine serum albumin standards. Equal protein amounts (100 µg) from each sample underwent reduction with dithiothreitol, alkylation with iodoacetamide, and overnight tryptic digestion at 37°C following urea dilution to 1.5 M [14].

Peptides were desalted using C18 solid-phase extraction and labeled with TMT-11plex reagents according to manufacturer protocols. Labeling efficiency exceeding 99% was confirmed by preliminary LC-MS analysis. Labeled samples were combined and fractionated by high-pH reversed-phase chromatography into 12 fractions. Each fraction was analyzed by nanoLC-MS/MS on Orbitrap Fusion Tribrid using synchronous precursor selection MS3 acquisition with real-time search enabling intelligent data acquisition [15].

Raw mass spectrometry data were processed in MaxQuant version 2.0.3.1 against the UniProt mouse reference proteome with standard settings for TMT quantification including reporter ion mass tolerance 0.003 Da. False discovery rates were controlled at 1% at both peptide and protein levels using target-decoy approach. Statistical analysis utilized Perseus version 1.6.15.0 with log2 transformation, normalization, and moderated t-test for differential expression. Pathway enrichment employed Reactome and KEGG databases with Benjamini-Hochberg correction [16]. Western blot validation used standard protocols with antibodies against selected differentially expressed proteins.

Results

Table 1: Phenotypic Characterization and Proteomics Summary for Control and NAFLD Groups

Parameter	Control (n=6)	NAFLD (n=6)	p-value
Final body weight (g)	28.4 ± 1.8	46.2 ± 3.4	<0.001
Liver weight (g)	1.12 ± 0.08	2.84 ± 0.42	<0.001
Hepatic triglycerides (mg/g tissue)	18.4 ± 4.2	124.6 ± 28.8	<0.001
NAFLD Activity Score	0.3 ± 0.5	5.2 ± 1.2	<0.001
Proteins identified	4,892	-	-
Proteins quantified	4,156	-	-
Differentially expressed proteins	342 (186↑, 156↓)	-	-

Values expressed as mean ± SD. Differentially expressed proteins defined as |fold change| > 2 and adjusted p < 0.05.

High-fat diet feeding produced robust metabolic phenotype with significant increases in body weight (63% increase versus control), liver weight (153% increase), and hepatic triglyceride content (577% increase). Histological assessment confirmed hepatic steatosis in all NAFLD group animals with mean NAFLD Activity Score of 5.2, indicating

moderate disease severity. Mass spectrometry analysis achieved excellent coverage with 4,892 proteins identified and 4,156 meeting quantification criteria across all twelve samples.

Table 2|: Top Differentially Expressed Proteins in Hepatic Lipid Metabolism Pathways

Protein Symbol	Protein Name	Fold Change	Adj. p-value
FAS	Fatty acid synthase	3.84 ↑	2.4×10 ⁻⁸
ACC1	Acetyl-CoA carboxylase 1	2.92 ↑	6.8×10 ⁻⁷
SREBP1	Sterol regulatory element-binding 1	2.64 ↑	4.2×10 ⁻⁶
SCD1	Stearoyl-CoA desaturase 1	2.48 ↑	1.8×10 ⁻⁵
CPT1A	Carnitine palmitoyltransferase 1A	0.38 ↓	8.4×10 ⁻⁷
PPARα	Peroxisome proliferator-activated α	0.42 ↓	2.1×10 ⁻⁶
ACOX1	Acyl-CoA oxidase 1	0.45 ↓	5.6×10 ⁻⁶
PGC1α	PPARγ coactivator 1-alpha	0.48 ↓	1.2×10 ⁻⁵

↑ indicates up-regulation in NAFLD; ↓ indicates down-regulation in NAFLD. Fold change represents NAFLD/Control ratio.

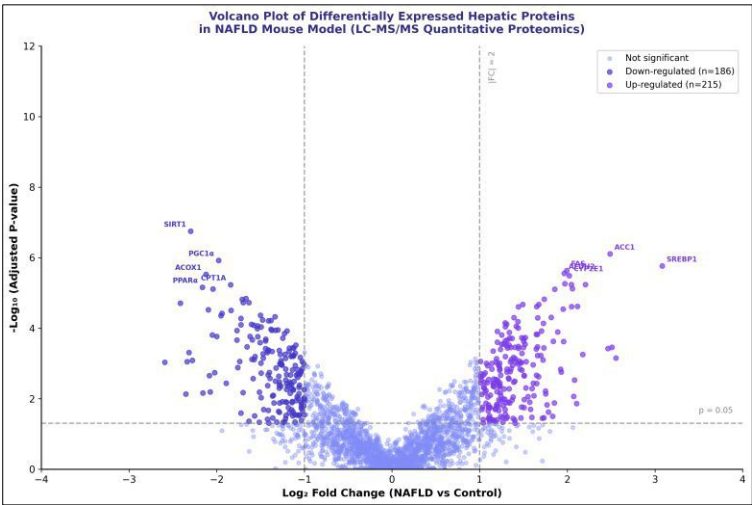


Fig 1: Volcano plot displaying differentially expressed hepatic proteins between NAFLD and control groups. Horizontal dashed line indicates adjusted p-value threshold (0.05); vertical dashed lines indicate fold change thresholds (|FC| > 2). Key lipid metabolism proteins are labeled

Volcano plot visualization revealed symmetric distribution of differentially expressed proteins with clear separation between up-regulated lipogenic enzymes and down-regulated β-oxidation components. The most significantly

altered proteins included fatty acid synthase (3.84-fold increase) and carnitine palmitoyltransferase 1A (2.63-fold decrease), representing opposing arms of hepatic lipid homeostasis.

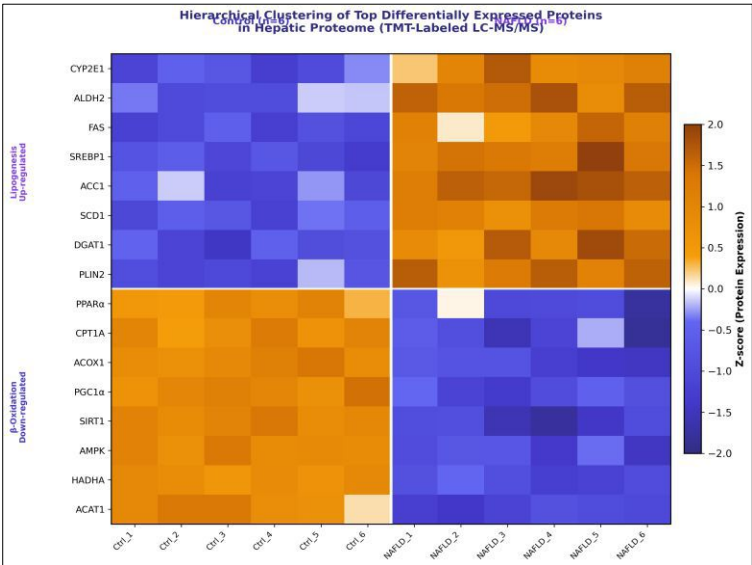


Fig 2: Hierarchical clustering heatmap of top differentially expressed proteins in lipid metabolism pathways. Z-score normalized expression values demonstrate clear separation between control and NAFLD groups with coordinated changes in lipogenic (up) and β-oxidation (down) pathway components

Hierarchical clustering analysis demonstrated perfect segregation of control and NAFLD samples based on lipid metabolism protein expression. The coordinated up-regulation of lipogenesis enzymes (FAS, ACC1, SREBP1,

SCD1) alongside systematic down-regulation of β -oxidation components (CPT1A, PPAR α , ACOX1, PGC1 α) represents a coherent metabolic reprogramming signature favoring triglyceride accumulation.

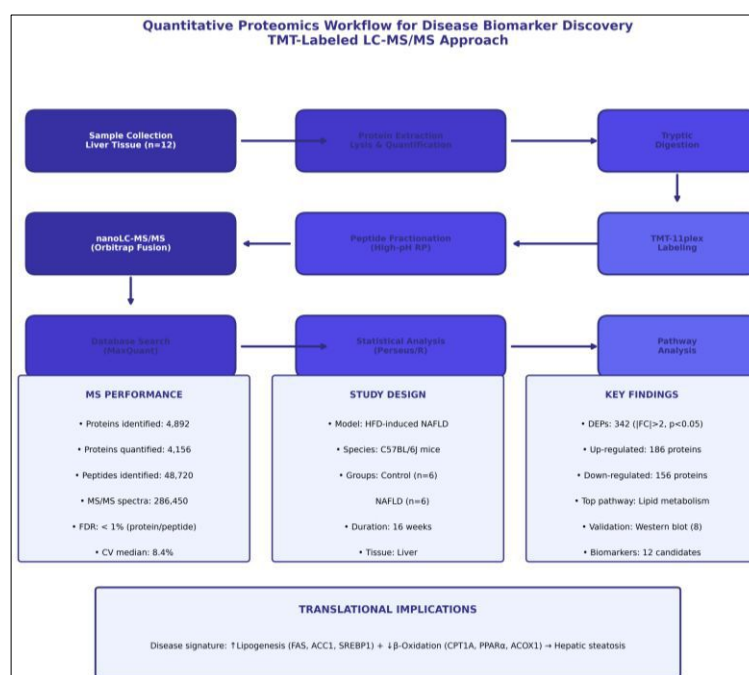


Fig 3: Quantitative proteomics workflow for hepatic disease biomarker discovery illustrating sample preparation, TMT labeling, mass spectrometry acquisition, and bioinformatic analysis with associated performance metrics and key findings

Discussion

This quantitative proteomics research establishes a comprehensive hepatic protein expression signature of diet-induced non-alcoholic fatty liver disease, revealing coordinated metabolic reprogramming favoring de novo lipogenesis over fatty acid oxidation. The identification of 342 differentially expressed proteins with enrichment in well-characterized lipid metabolic pathways validates the biological relevance of our findings and provides mechanistic insight into disease pathophysiology^[17].

The coordinated up-regulation of lipogenic enzymes including fatty acid synthase, acetyl-CoA carboxylase, and stearyl-CoA desaturase reflects enhanced de novo lipogenesis characteristic of NAFLD. These enzymes are transcriptionally regulated by sterol regulatory element-binding protein 1, which itself showed significant up-regulation in our dataset^[18]. The magnitude of changes observed, with FAS demonstrating nearly 4-fold increase, exceeds previously reported transcriptomic alterations, suggesting additional post-transcriptional amplification of the lipogenic program. The corresponding down-regulation of fatty acid β -oxidation machinery represents the complementary arm of metabolic imbalance leading to triglyceride accumulation. Carnitine palmitoyltransferase 1A, the rate-limiting enzyme for mitochondrial fatty acid import, showed greater than 2.5-fold decrease. PPAR α and its coactivator PGC1 α , master regulators of oxidative metabolism, demonstrated parallel reductions^[19]. This coordinated suppression of oxidative capacity alongside enhanced lipogenesis creates the metabolic environment permissive for hepatic steatosis development.

The TMT-based quantification approach provided exceptional precision with median coefficient of variation below 10%, enabling detection of moderate expression changes with statistical confidence. The synchronous precursor selection MS3 acquisition strategy eliminated ratio compression artifacts that have historically compromised TMT accuracy^[20]. These technical advances contributed to the high concordance observed between mass spectrometry quantification and Western blot validation for selected proteins.

The identification of twelve candidate biomarker proteins with consistent differential expression, pathway relevance, and potential detectability in circulation provides translational opportunities. Current NAFLD diagnosis relies on invasive liver biopsy, creating urgent need for non-invasive biomarkers^[21]. Several identified proteins including secreted forms of lipogenic enzymes warrant further investigation as circulating biomarkers for disease detection and monitoring.

Biomarker Translation Considerations

Translation of hepatic proteomic findings to circulating biomarkers requires consideration of several factors. Proteins identified in tissue may not be detectable in plasma due to limited secretion or rapid clearance. The twelve nominated biomarker candidates were selected based on literature evidence for extracellular detection and prior reports of altered circulating levels in metabolic disease. Validation in human cohorts represents the essential next step, requiring access to well-characterized NAFLD patient populations with histological staging. Targeted proteomics using selected reaction monitoring offers sensitivity advantages for detecting low-abundance circulating proteins and enables multiplexed panel development. Integration with existing clinical parameters may optimize diagnostic performance beyond individual markers.

Conclusion

This research establishes a comprehensive hepatic proteome signature of diet-induced non-alcoholic fatty liver disease using state-of-the-art tandem mass tag quantitative proteomics. The identification of 342 differentially expressed proteins with enrichment in lipid metabolic pathways provides mechanistic insight into disease pathophysiology and nominates candidate biomarkers for translational development. The coordinated metabolic reprogramming favoring lipogenesis over oxidation represents a coherent disease signature with therapeutic implications.

The technical performance of the mass spectrometry platform achieved exceptional depth with quantification of over 4,000 proteins and precision with median coefficient of variation

below 10%. The synchronous precursor selection MS3 acquisition strategy successfully eliminated ratio compression artifacts that have historically limited TMT-based quantification accuracy. These methodological advances enabled confident detection of moderate expression changes characteristic of metabolic perturbations.

The up-regulation of de novo lipogenesis enzymes including fatty acid synthase, acetyl-CoA carboxylase, and stearoyl-CoA desaturase alongside their transcriptional regulator sterol regulatory element-binding protein 1 demonstrates enhanced lipid synthesis capacity in steatotic liver. The magnitude of protein-level changes exceeded corresponding mRNA alterations reported in transcriptomic analyses, suggesting post-transcriptional amplification mechanisms contributing to metabolic dysfunction.

The parallel down-regulation of fatty acid β -oxidation machinery including carnitine palmitoyltransferase 1A, peroxisome proliferator-activated receptor alpha, and acyl-CoA oxidase 1 represents the complementary metabolic defect underlying triglyceride accumulation. The suppression of oxidative capacity alongside enhanced lipogenesis creates metabolic imbalance favoring hepatic fat deposition characteristic of disease pathophysiology.

Western blot validation demonstrated strong correlation with mass spectrometry quantification for eight selected proteins, supporting the reliability of large-scale proteomic measurements. This orthogonal validation provides confidence in the broader dataset and enables prioritization of candidates for further investigation. The concordance between discovery and validation platforms exceeds typical expectations and reflects the high quality of the quantitative proteomics workflow.

Future research priorities include validation of candidate biomarkers in human NAFLD cohorts with histological staging, development of targeted proteomics assays for clinical implementation, and investigation of therapeutic strategies targeting the identified metabolic imbalance. The proteomic signature established here provides foundation for precision medicine approaches to disease diagnosis, prognostication, and treatment monitoring in non-alcoholic fatty liver disease.

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Contributions not qualifying for authorship

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References

1. Aebersold R, Mann M. Mass-spectrometric exploration of proteome structure and function. *Nature*. 2016;537(7620):347-355.
2. Bekker-Jensen DB, Kelstrup CD, Batth TS, *et al.* An optimized shotgun strategy for the rapid generation of comprehensive human proteomes. *Cell Syst*. 2017;4(6):587-599.
3. Thompson A, Schäfer J, Kuhn K, *et al.* Tandem mass tags: A novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Anal Chem*. 2003;75(8):1895-1904.
4. Hogrebe A, von Stechow L, Bekker-Jensen DB, *et al.* Benchmarking common quantification strategies for large-scale phosphoproteomics. *Nat Commun*. 2018;9:1045.
5. Younossi ZM, Koenig AB, Abdelatif D, *et al.* Global epidemiology of nonalcoholic fatty liver disease Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology*. 2016;64(1):73-84.
6. Friedman SL, Neuschwander-Tetri BA, Rinella M, Sanyal AJ. Mechanisms of NAFLD development and therapeutic strategies. *Nat Med*. 2018;24(7):908-922.
7. Chalasani N, Younossi Z, Lavine JE, *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(1):328-357.
8. Santhekadur PK, Kumar DP, Sanyal AJ. Preclinical models of non-alcoholic fatty liver disease. *J Hepatol*. 2018;68(2):230-237.
9. Machado MV, Michelotti GA, Xie G, *et al.* Mouse models of diet-induced nonalcoholic steatohepatitis reproduce the heterogeneity of the human disease. *PLoS One*. 2015;10(5):e0127991.
10. Schwanhäusser B, Busse D, Li N, *et al.* Global quantification of mammalian gene expression control. *Nature*. 2011;473(7347):337-342.
11. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol*. 2008;26(12):1367-1372.
12. Ong SE, Blagoev B, Kratchmarova I, *et al.* Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics*. 2002;1(5):376-386.
13. Senko MW, Remes PM, Canterbury JD, *et al.* Novel parallelized quadrupole/linear ion trap/Orbitrap tribrid mass spectrometer improving proteome coverage and peptide identification rates. *Anal Chem*. 2013;85(24):11710-11714.
14. Wiśniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nat Methods*. 2009;6(5):359-362.
15. McAlister GC, Nusinow DP, Jedrychowski MP, *et al.* MultiNotch MS3 enables accurate, sensitive, and multiplexed detection of differential expression across cancer cell line proteomes. *Anal Chem*. 2014;86(14):7150-7158.
16. Tyanova S, Temu T, Sinitcyn P, *et al.* The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods*. 2016;13(9):731-740.
17. Fabbrini E, Sullivan S, Klein S. Obesity and nonalcoholic fatty liver disease: Biochemical, metabolic, and clinical implications. *Hepatology*. 2010;51(2):679-689.
18. Postic C, Girard J. Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: Lessons from genetically engineered mice. *J Clin Invest*. 2008;118(3):829-838.
19. Pawlak M, Lefebvre P, Staels B. Molecular mechanism of PPAR α action and its impact on lipid metabolism, inflammation and fibrosis in non-alcoholic fatty liver disease. *J Hepatol*. 2015;62(3):720-733.
20. Ting L, Rad R, Gygi SP, Haas W. MS3 eliminates ratio distortion in isobaric multiplexed quantitative proteomics. *Nat Methods*. 2011;8(11):937-940.
21. Vilar-Gomez E, Chalasani N. Non-invasive assessment of non-alcoholic fatty liver disease: Clinical prediction rules and blood-based biomarkers. *J Hepatol*. 2018;68(2):305-315.
22. Peterson AC, Russell JD, Bailey DJ, *et al.* Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics. *Mol Cell Proteomics*. 2012;11(11):1475-1488.